VOLUME 30 APRIL 1952 NUMBER 2

Canadian Journal of Medical Sciences

Editor: J. B. COLLIP

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF MEDICAL SCIENCES

(Formerly Section E, Canadian Journal of Research)

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Journal of Medical Sciences

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A QUANTITATIVE METHOD FOR THE *IN VITRO* ASSAY OF FUNGISTATIC AGENTS[1]

BY F. BLANK

Abstract

A simple and practical filter paper strip method for the *in vitro* assay of fungistatic agents (substances and ointments) has been described. The method involves a modified diffusion technique and can be applied to both water soluble and water insoluble compounds. It is sensitive, contamination is rare, and both pathogenic and nonpathogenic fungi can be used as test organisms. Several organisms can be tested simultaneously. The results are quantitative and reproducible.

Introduction

The increasing number of cases of dermatomycosis (21, 32) stimulated a search for new antimycotics. As the most commonly occurring dermatophytes, *Ctenomyces interdigitalis* (Priestley) Langeron et Milochevitch* (Syn. *Trichophyton interdigitale* Priestley) and *Trichophyton rubrum* (Cast.) Sabouraud (2, 4, 15, 20, 21, 27, 29, 31, 37) cannot be transmitted to laboratory animals (3, 26, 27, 29), reliable *in vitro* tests for the substances supposed to be antimycotics must be used before any clinical experiments can be made. Numerous methods have therefore been devised for the *in vitro* assay of fungistatic compounds (1, 4, 5, 7, 10, 11, 12, 14, 17, 22, 23, 24, 25, 28, 30, 33, 34, 35, 36).

The classic dilution method is more complicated for the assay of fungistatic agents than for bacteriostatic compounds. We have therefore developed a new method for this purpose which is based on a technique described by Hirsch (13) for the evaluation of bacteriostatic agents. Although designed for the use of dermatophytes and pathogenic yeasts as test organisms it can also be performed with other fungi such as *Actinomycetes*, *Aspergillus-*, *Cephalosporium-*, *Cladosporium-*, *Hormodendrum-*, *Penicillium-*, *Sporotrichum-*, and other species. The assay can be carried out simply and quickly and gives quantitative and reproducible results. Moreover, it permits the performance of a series of tests simultaneously.

[1] *Manuscript received January 31, 1952.*

Contribution from the Department of Bacteriology and Immunology, McGill University, Montreal, Que.

**The systematics of Langeron and Milochevitch (19) will be used for the dermatophytes.*

The Filter Paper Strip Method

a. Culture Media

An appropriate medium for testing compounds against dermatophytes should meet the following two requirements:

1. The culture medium must guarantee an optimal growth of the fungus.

2. Some substances show fungistatic activity *in vitro* but are inactivated in the presence of body fluids or animal tissues. The addition of serum to the culture medium is, therefore, essential in order to avoid misleading results (6, 16).

The following quantitative technique was used for the examination of the composition of the culture medium.

We found by quantitative experiments that dilute watery solutions of Tween 80 did not affect the growth of fungi. Therefore, one to two drops of Tween 80 are put into an Erlenmeyer flask containing 200 cc. of sterile distilled water. This solution is examined 24 hr. before its use for sterility by means of blood agar plates. Five cubic centimeters of this Tween 80 solution are poured into a test tube which contains a three-weeks-old culture of *Ctenomyces interdigitalis* on Sabouraud glucose agar "Difco". The test tube is then rolled several times between the hands and the suspension of thallospores and mycelium is decanted into a sterile test tube. Twenty-five to forty Petri dishes, each containing 20 cc. of the medium to be investigated, are inoculated with 0.8 cc. of this suspension. The inoculum must be equally distributed over the whole medium by agitating the covered Petri dishes gently. The Petri dishes are then incubated. After one week, cultures showing contamination are eliminated and the remaining cultures are killed by autoclaving at 121°C. for one hour. Before cooling, the whole mycelium is taken from the still liquid medium and is carefully washed several times with hot water. Finally, the mycelium is put on a Buchner funnel, washed with distilled water, dried at 105-110°C. for three hours and weighed. By means of this technique different peptones and carbohydrates together with extracts, growth substances, protein hydrolyzates, and mineral salts were examined as to their suitability for a culture medium guaranteeing an optimal growth of dermatophytes. Our experiments resulted in the following medium, which represents a modified Sabouraud medium:

2% Neopeptone "Difco" or peptone bactériologique "Uclaf",
4% maltose "Wander",
0.1% yeast extract "Difco",
10% bovine serum,
2.5% agar in tap water.

The importance of adding serum (10%) to this medium is demonstrated by the following experiment (see Table I).

Using *Candida* species as test organisms the following medium was found suitable (18): 2% crude glucose, 1% Neopeptone "Difco", 10% bovine serum, 2.5% agar in tap water.

TABLE I

WEIGHT OF THE DRIED MYCELIUM OF *Ctenomyces interdigitalis* IN MGM. GROWN DURING EIGHT DAYS ON THE MODIFIED SABOURAUD MEDIUM AT 20°C.

Serum added	Without serum
287	92
274	93
265	103
238	102
263	101
214	101
215	128
240	103
249	104
263	123
246	123
230	98
277	108
221	144
277	124
233	123
231	108
	139
	120
248.4 ± 5.6	112.8 ± 3.4

b. Temperature

Gabrielsen (8) found by quantitative measurements of growing cultures of *Ctenomyces interdigitalis* that optimal growth occurred at 30°C. Similar results were obtained by Giblett and Henry (9) working with *Sabouraudites Saudouini* (Syn., *Microsporum audouini*), *S. canis* and *S. gypseus*. Therefore, all our *in vitro* tests were performed at this temperature both with *Ctenomyces interdigitalis* and all other dermatophytes for which the optimal temperature has not yet been determined. Tests with *Candida* species were performed at 25°C.

c. Inocula

Owing to the more complicated morphology of the fungi, streaks cannot be made with them as easily as with bacteria (13). The following technique for the performance of streaks with fungi was adopted. Suspensions of thallospores and mycelium of species of the genus *Ctenomyces* are prepared in the manner described above using Tween 80. In the preparation of suspensions of thallospores and mycelium of *Trichophyton rubrum*, *T. Sabouraudi*, *T. Megnini*, *T. epilans*, *T. tonsurans*, *T. sulfureum*, *T. ferrugineum*, *Sabouraudites audouini*, *S. canis*, *S. gypseus*, and *Epidermophyton floccosum* the surface of the culture is scraped several times with a hooked stainless steel needle. Inocula for streaks with *Candida* species are taken from cultures in liquid media (2% crude glucose, 1% Neopeptone "Difco" or peptone bactériologique "Uclaf") grown during 48 hr. at 25°C.

d. Solvents

The best solvent for water insoluble compounds is methylcellosolve (2-methoxyethanol). The largest inhibition zones of any compound were found if methylcellosolve was used as a vehicle. It seems that methylcellosolve increases the diffusion rate of the compound tested. *p*-Dioxane and ethanol can also be used.

e. Concentration of the Solutions to be Tested

Usually, we begin with $M/10$ and $M/50$ solutions of known compounds supposed to be fungistatic. Effective fungistatic agents should have an inhibition zone of 40 to 50 mm. if examined in $M/50$ solutions.

f. Technique of the Test

As mentioned under (*a*), 20 cc. of the culture medium are poured into sterile Petri dishes. Sterile filter paper strips (85 mm. long, 5 mm. wide) bearing paper clips at one end are dipped into the solutions of the compounds to be tested. The wet strips are then suspended from nails fastened to a ledge. About 25 to 30 cu. mm. of the solution are retained by each strip. If methylcellosolve or water has been used as a solvent the strips are quickly pressed between two sterile filter papers. The dried filter paper strips are extracted from the clips and then put across the center of the culture medium.

Filter paper strips dipped into the solvent only are used as controls. Thin cardboard strips are used for the assay of ointments. With a sterile wooden toothpick some ointment is put on one side of the strip. By drawing the strip through two sterile filter paper sheets the ointment is equally spread upon the cardboard. The medicated side is put on the medium. The Petri dishes are incubated at 37°C. for about 16 hr. to allow the test substance to diffuse into the medium. The next day the dishes are inoculated. A loop is dipped into the inoculum and a streak is drawn from the border of the Petri dish to the filter paper strip. The same is done on the opposite half of the Petri dish at the same height. Four corresponding streaks 2 cm. from each other can be drawn on each half of the Petri dish. Thus, four different strains of a species, or four different species, can be tested on one dish, provided that there is no growth inhibiting interaction between them. The inoculated Petri dishes are incubated at 30°C. The size of the inhibition zones, as measured by the distance of the two corresponding fungus streaks, gives the result of the assay (see Fig. 1). The inhibition zones are measured on the bottom of the Petri dish on three consecutive days. The date of reading depends upon the speed of growth of the species used. Test species of the genus *Ctenomyces*, likewise assays with *Trichophyton rubrum*, are read the third, fourth, and fifth days after inoculation. The tests with *Candida* species are measured 24 and 48 hr. after inoculation.

Discussion

The method described has two disadvantages. First, some dermatophytes such as *Trichophyton Schönleini*, *T. violaceum*, *T. glabrum*, and *T. concentricum* may not be used satisfactorily as test organisms because of their slow growth

PLATE I

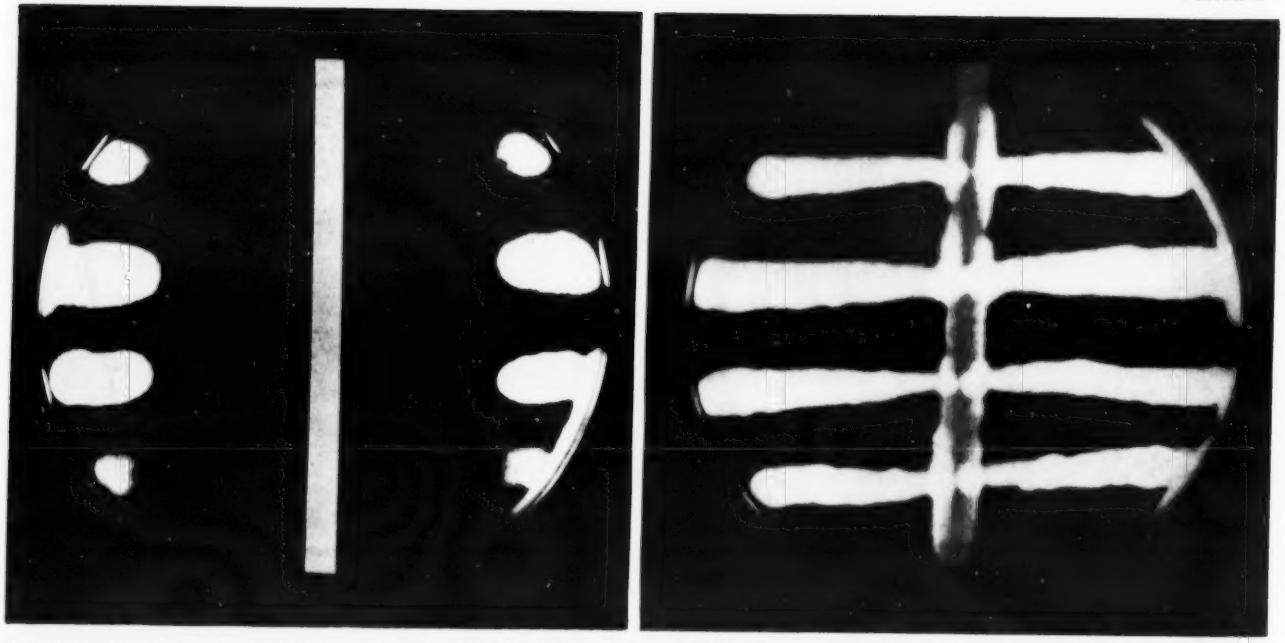

FIG. 1. Filter paper strip method. Test organism: *C. interdigitalis*. Left: Fungistatic effect of ½ *M* sodium caprylate. Right: Control (solvent).

and the difficulty of making suitable suspensions. It may be assumed, however, that an effective fungistatic agent inhibiting the growth of *Ctenomyces interdigitalis* or *Trichophyton rubrum* would also affect the growth of these organisms.

The second criticism leveled at this method is that leveled at all methods based on diffusion: the inhibition zones depend on the rate of diffusion into the culture medium. However, it is not conceivable that a substance which does not spread into the culture medium would affect the fungus growing in skin, nails, or hairs.

References

1. Barail, L. C. Am. Dyestuff Reptr. 37: 257. 1948.
2. Blank, F. Dermatologica, 102: 88. 1951.
3. Blank, F. and Schuppli, R. Dermatologica, 102: 102. 1951.
4. Burke, R. C. and Bumgarner, F. E. Arch. Dermatol. Syphilol. 60: 742. 1949.
5. Burlingame, E. M. and Reddish, G. F. J. Lab. Clin. Med. 24: 765. 1939.
6. Council on Pharmacy and Chemistry. American Medical Association. J. Am. Med. Assoc. 121: 593. 1943.
7. Emmons, C. W. Am. J. Pub. Health, 35: 844. 1945.
8. Gabrielsen, E. K. Acta Dermato-Venereol. 23: 405. 1943.
9. Giblett, E. R. and Henry, B. S. J. Investigative Dermatol. 14: 377. 1950.
10. Golden, M. J. and Oster, K. A. J. Am. Pharm. Assoc., Sci. Ed. 36: 283. 1947.
11. Grunberg, E. Yale J. Biol. Med. 19: 855. 1947.
12. Hillegas, A. B. and Camp, E. J. Investigative Dermatol. 6: 217. 1945.
13. Hirsch, J. Monatsschr. Kinderheilk. 99: 5. 1951.
14. Horsfall, J. G. Botan. Rev. 11: 357. 1945.
15. Kaufmann-Wolf, M. Dermatol. Z. 21: 385. 1914.
16. Klarmann, E. G. Ann. New York Acad. Sci. 53: 123. 1950.
17. Kligman, A. M. and Rosenzweig, W. J. Investigative Dermatol. 10: 51. 1948.
18. Langeron, M. and Guerra, P. Ann. parasitol. humaine et comparée, 16: 36. 1938.
19. Langeron, M. and Milochevitch, S. Ann. parasitol. humaine et comparée, 8: 465. 1930.
20. Legge, R. T., Bonar, L., and Templeton, H. J. Arch. Dermatol. Syphilol. 27: 12. 1933.
21. Lomholt, S. Med. Klin. 34: 118. 1938.
22. Nickerson, W. J. Science, 103: 484. 1946.
23. Oster, K. A. and Golden, M. J. J. Am. Pharm. Assoc., Sci. Ed. 36: 359. 1947.
24. Oster, K. A. and Golden, M. J. J. Am. Pharm. Assoc., Sci. Ed. 37: 429. 1948.
25. Oster, K. A. and Golden, M. J. Am. J. Pharm. 121: 375. 1949.
26. Ota, M. and Kawatsuré, S. Ann. parasitol. humaine et comparée, 11: 206. 1933.
27. Ota, M. and Kawatsuré, S. Ann. parasitol. humaine et comparée, 11: 476. 1933.
28. Peck, S. M. Trans. N.Y. Acad. Sci. 11: 112. 1949.
29. Priestley, H. Med. J. Australia, 2: 471. 1917.
30. Reddish, G. F. Ann. N.Y. Acad. Sci. 53: 149. 1950.
31. Rivalier, E. Paris Medical, 95: 66. 1935.
32. Salvin, S. B. Ann. N.Y. Acad. Sci. 50: 1217. 1950.
33. Schraufstätter, E., Richter, R., and Ditscheid, W. Arch. Dermatol. u. Syphilis, 188: 259. 1949.
34. Sharlit, H. and Muskatblit, E. Arch. Dermatol. Syphilol. 25: 615. 1932.
35. Vanbreuseghem, R. Arch. Belges Dermatol. Syphilol. 6 (IV): 1. 1950.
36. Weidman, F. D., Emmons, C. W., Hopkins, J. G., and Lewis, G. M. J. Am. Med. Assoc. 128: 805. 1945.
37. Zündel, W. Arch. Dermatol. u. Syphilis, 179: 1. 1939.

STUDIES ON HEPATIC NECROSIS INDUCED BY DIETARY MEANS

V. THE INFLUENCE OF THYROID ACTIVITY ON THE PRODUCTION OF HEPATIC NECROSIS BY DIETARY MEANS[1]

By John R. McLean and J. M. R. Beveridge

Abstract

The addition of the antithyroid agents, thiocyanate and propylthiouracil, to a basal diet low in the sulphur-containing amino acids and in α-tocopherol ameliorated its necrogenic effect, but did not prevent the development of acute massive liver necrosis when this ration was fed to young rats. The necrogenic effect of a basal diet supplemented with desiccated thyroid, an agent that greatly potentiates the necrogenicity of the ration, was completely abolished by the addition of cystine or α-tocopherol. These substances did not overcome the well known toxic effects of excessive amounts of thyroid tissue.

Introduction

Although there is no complete agreement amongst clinicians on the role of hyperthyroidism in liver dysfunction, it would appear that the majority of reports support the belief that there is in fact a relationship between these two conditions (14, 16, 17). In the experimental field there is even less unanimity of opinion regarding the role of thyrotoxicosis in liver damage. For example, Davis and Whipple (8) reported that excessive amounts of thyroid did not potentiate the action of hepatoxic agents, such as chloroform, on the livers of dogs, a finding later confirmed in rats by Cameron and Karunaratne (5). However, a conflicting finding was reported by McIver (15) who concluded that rats maintained on a standard diet and injected with crystalline thyroxine, or fed desiccated thyroid tissue, showed an increased susceptibility to chloroform poisoning. A decreased level of thyroid activity brought about by thyroidectomy or the administration of antithyroid drugs has been claimed to prevent or retard the development of liver cirrhosis (10).

Handler and Follis (11) have shown that acute massive liver necrosis, a lesion that develops in rats on a diet deficient in the sulphur-containing amino acids (6) and low in α-tocopherol (12, 9), occurs more readily in hyperthyroid rats. This work has been confirmed by Abell and Beveridge (2). Handler and Follis also reported that thiouracil exerted a prophylactic action.

The fact that thyrotoxicosis was found to increase the necrogenicity of diets low in α-tocopherol and in the sulphur-containing amino acids suggested the possibility that the thyroid gland might play an integral role in the pathogenesis of acute massive liver necrosis. In this connection it is of interest to note that both methionine and cystine have been reported to prevent the decrease in liver glycogen seen in experimental hyperthyroidism (1). Furthermore, Houssay (13) has shown that a lowered metabolic rate, induced by thyroidectomy or administration of thiouracil, caused an increase in free

[1] *Manuscript received November 29, 1951.*
Contribution from the Department of Biochemistry, Queen's University, Kingston, Ont.

sulphydryl groups in the tissue of rats, a finding that again draws attention to a possible relationship between thyroid activity and the sulphur-containing amino acids.

In view of the relationships outlined, it was considered worthwhile to investigate further the effects of desiccated thyroid and antithyroid agents on the development of liver necrosis. According to Astwood (4) there are two large classes of antithyroid agents: those whose action can be overcome by administration of sufficient iodine; and those whose action is unaffected by this treatment. Potassium thiocyanate was chosen as a member of the former group and propylthiouracil as a member of the latter group.

Methods

Forty male rats of the Sprague–Dawley strain were divided into four groups of 10 rats of average initial weight, 54 gm. (S.D. 6.4). These were housed in individual cages with $\frac{1}{3}$ in. mesh screen floors and daily were offered approximately 8 gm. of the test diets, the basal composition of which is shown in Table I. The experiment was terminated at 105 days.

TABLE I

COMPOSITION OF BASAL DIET

Dietary ingredient	Per cent
Yeast (Fleischmann's active dried bakers' yeast)	18
Cornstarch	69
Lard	5
Salt mixture*	3
Sugar vitamin mixture**	1
Cod liver oil (Mead Johnson Company 3000 I.U. of vitamin A and 400 I U of vitamin D per gram)	2
Celluflour	2

*Himsworth's salt mixture. (Personal communication, for details see Abell *et al.* (3).)

**The sugar vitamin mixture contributed the following amounts of B vitamins per 8 gm. of food: thiamine hydrochloride, 20 μgm.; riboflavin, 25 μgm.; pyridoxine hydrochloride, 20 μgm.; calcium pantothenate, 100 μgm.

The presence or absence of liver necrosis was based upon an examination of histological sections from the three main lobes of the liver stained with haematoxylin and eosin. Similar sections were made of the thyroid tissue.

Results and Discussion

The effects of dietary supplements on the incidence and time required for development of liver necrosis are shown in Table II.

The data given here for Groups *B* and *T* are taken from a previous experiment, performed by one of us (J.M.R.B.) in collaboration with Abell (2), in which the same basal diet was used. The variations in incidence and time required for development of liver necrosis in weanling rats fed Diet *B* on three separate occasions were very small (incidence: 95%, 90%, 100%; average

TABLE II

THE EFFECT OF DIETARY SUPPLEMENTS ON THE DEVELOPMENT OF LIVER NECROSIS

Group*	Dietary supplement	Rats dying of necrosis or killed in extremis		Rats dying of other causes		Total incidence of necrosis, corr'd.**	
		Number rats	Av. days on diet	Number rats	Av. days on diet	Number	%
B	None	9	39	1	42	9/10	90
T	0.3% Desiccated thyroid	7	20	3	13	7/8	88
P	0.5% Propylthiouracil	2	59	5	55	3/10***	30
CN	0.3% Potassium thyiocyanate	8	56	2	69	8/10	80
TC	0.3% Desiccated thyroid +0.5% L-cystine	0	—	8	57	0	0
TE	0.3% Desiccated thyroid +2 mgm. α-tocopherol acetate per 8 gm. diet	0	—	9	43	0	0

**10 rats per group.*

***Rats that died of causes other than liver necrosis were eliminated from consideration if they died prior to the time at which the first death due to liver necrosis occurred in the remainder of the group. This convention had to be applied in the case of Group T only.*

****One animal dying at 66 days showed necrosis in a small part of one lobe of the liver and it was believed that this lesion was insufficient to have caused the death of the animal.*

times at which liver necrosis developed: 38, 39, 41 days). It was therefore felt that valid comparisons could be made between the data on Groups *B* and *T* and the results obtained here.

The prophylactic action of propylthiouracil is demonstrated by a comparison of Groups *P* and *B*. Only three out of 10 animals in Group *P* developed necrosis, whereas the incidence of necrosis in Group *B* was 90%. Propylthiouracil, however, cannot be classed as having a strong antinecrogenic action. In a previous experiment performed by Abell and Beveridge (2), it was found that although 0.3% propylthiouracil prevented the development of liver necrosis, the interpretation of this experiment was complicated by the fact that the animals succumbed after an average period of 47 days. In the experiment herein reported the antithyroid agent was fed at a level of only 0.05%. This level was sufficiently low to permit three of the animals to survive the experiment, but allowed lethal liver necrosis to develop in two of the remainder of the group. The fact that five of the group died at an average period of 55 days, presumably as a result of the toxic effect of the drug, indicates that any higher level would not have yielded a greater beneficial action. The thyroid tissue of the animals fed propylthiouracil presented, both grossly and microscopically, the hyperplastic appearance characteristic of the administration of this goitrogenic agent.

The addition of potassium thiocyanate to the basal diet increased from 39 to 56 days the time required for death due to liver necrosis to occur. The thyroid tissues of this group showed some degree of hyperplasia which, however,

was much less marked than that seen in the group supplemented with propylthiouracil.

The addition of cystine to a diet containing desiccated thyroid prevented the development of liver necrosis and enabled the animals to survive for an average period of 57 days. Indeed, two animals survived the experimental period of 105 days.

The supplementation of Diet *T* with α-tocopherol also prevented the development of liver necrosis and enabled the animals to survive for an average period of 43 days, one animal surviving the experimental period of 105 days.

The liver sections from Groups *TE* and *TC* showed some degenerating nuclei and marked albuminous degeneration but the typical picture of acute massive necrosis was absent. The thyroid tissues of these groups presented the characteristic hypoplastic appearance of animals fed desiccated thyroid.

It has been observed that rats maintained on our necrogenic diet show a variable degree of blanching of the incisor teeth, particularly the upper incisors. This has been described by Davies and Moore (7) as a sign of a vitamin E deficiency. In the animals receiving desiccated thyroid this bleaching was most noticeable, the teeth being completely devoid of pigment. It may be of interest to note that the addition of α-tocopherol along with desiccated thyroid had no effect on the degree of depigmentation. No explanation for this phenomenon can be offered at present.

The fact that α-tocopherol and cystine prevented liver necrosis in hyperthyroid animals leads to the conclusion that a high metabolic rate per se is not a prime factor in the development of liver necrosis. It would appear that the level of thyroid activity is a subsidiary, not a determining factor, in the production or prevention of this hepatic lesion.

Acknowledgments

This investigation was supported by a grant from the National Research Council of Canada.

References

1. ABELIN, I. Helv. Physiol. et Pharmacol. Acta, 3: 481. 1945.
2. ABELL, M. R. and BEVERIDGE, J. M. R. Arch. Path. 52: 428. 1951.
3. ABELL, M. R., BEVERIDGE, J. M. R., and FISHER, J. H. Arch. Path. 50: 1. 1950.
4. ASTWOOD, E. B. J. Pharmacol. Exptl. Therap. 78: 79. 1943.
5. CAMERON, G. R. and KARUNARATNE, W. A. E. J. Path. Bact. 41: 267. 1935.
6. DAFT, F. S., SEBRELL, W. H., and LILLIE, R. D. Proc. Soc. Exptl. Biol. Med. 50: 1. 1942.
7. DAVIES, A. W. and MOORE, T. Nature, 147: 794. 1941.
8. DAVIS, N. C. and WHIPPLE, G. H. Arch. Internal Med. 23: 612. 1919.
9. GYORGY, P. and GOLDBLATT, H. J. Exptl. Med. 89: 245. 1949.
10. GYORGY, P., ROSE, C. S., and GOLDBLATT, H. Proc. Soc. Exptl. Biol. Med. 67: 67. 1948.
11. HANDLER, P. and FOLLIS, R. H. J. Nutrition, 35: 669. 1948.
12. HIMSWORTH, H. P. and LINDAN, O. Nature, 163: 30. 1949.
13. HOUSSAY, A. B. Rev. soc. argentina biol. 24: 87. 1948.
14. LICHTMAN, S. S. Ann. Internal Med. 14: 1199. 1941.
15. McIVER, M. A. Proc. Soc. Exptl. Biol. Med. 45: 201. 1940.
16. MADDOCK, W. G., PEDERSON, S., and COLLER, F. J. Am. Med. Assoc. 109: 2130. 1937.
17. YOUMANS, J. B. and WARFIELD, L. M. Arch. Internal Med. 37: 1. 1926.

THE INFLUENCE OF HEPARIN ON THE ACTIVATION ENERGY OF THROMBIN INACTIVATION[1]

By John J. Spitzer[2]

Abstract

Gerandas's method for thrombin inactivation was used in these experiments. The activation energy of thrombin inactivation was calculated and found to be about 9000-14,000 cal. per mole. Heparin *in vitro* decreases the activation energy of thrombin inactivation, indicating that its action is the same as that of a catalyst.

Introduction

In 1892 Schmidt (12) first described the capacity of blood serum to destroy thrombin. This capacity is due to antithrombic substances of serum. Ample bibliography of the literature of antithrombins is to be found in the papers of Volkert (16), Quick (11), Glazko and Ferguson (9, 10), and Seegers (13), among others.

Opinions on the nature of antithrombins are different. Many authors considered the antithrombins as one system (which can be influenced by heparin), others, like Astrup and Darling (1, 2, 3), found that there are two antithrombin systems. One of them is an antithrombin by itself, the other acts as an antithrombin only in the presence of heparin.

All the authors agree that heparin increases the antithrombic power of the blood. The mechanism of action of heparin in the inactivation of thrombin is not known. Seegers, Warner, Brinkhous, and Smith (15), and Seegers and Smith (14) presumed that the reaction was an enzymatic one. This hypothesis, however, was not proved.

The purpose of this paper is (1) to determine the activation energy of thrombin inactivation and (2) to study the nature of heparin action on the basis of its influence on the activation energy.

Methods

For the determination of antithrombin activity, Gerendas's method of thrombin inactivation was used (4, 5, 6, 7). Gerendas stated that thrombin inactivation is a reaction of the first order. His method is briefly the following: one brings together equal amounts of thrombin solution (of known strength) and serum. The strength of thrombin is then tested after one-half, one, two, three, and five minutes, by adding it to a fibrinogen solution and measuring the coagulation time. These coagulation time values are plotted

[1] *Manuscript received November 12, 1951.*
Contribution from the Department of Physiology, University of Budapest, Hungary.

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against the time (one-half, one, two, three, and five minutes) on a semilogarithmic paper and the velocity coefficient of the procedure is calculated from the slope of the curve.

Results

The velocity coefficient (K) was determined in each case at different temperatures and from these values the activation energy of the reaction was calculated on the basis of Arrhenius' equation (8, 17):

$$E=\frac{2.303\ (\log K_2-\log K_1)R}{\frac{1}{T_1}-\frac{1}{T_2}}.$$

Where E is the activation energy, K_1 is the velocity coefficient at T_1 temperature, K_2 is the velocity coefficient at T_2 temperature, and R is the gas constant and equals 1.98 cal.

The "Normal" values of Table I show that the activation energy of thrombin inactivation (E) is about 9000-14,000 cal. per mole.

TABLE I

ACTIVATION ENERGIES OF THROMBIN INACTIVATION

Expt. No. 1 (Rabbit)	Normal	E=9850 cal. per mole
	8 γ per cc. heparin (*in vitro*)	E=7760 cal. per mole
Expt. No. 2 (Cow)	Normal	E=10,350 cal. per mole
	2 γ per cc. heparin (*in vitro*)	E= 8100 cal. per mole
	4 γ per cc. heparin (*in vitro*)	E= 7500 cal. per mole
	8 γ per cc. heparin (*in vitro*)	E= 6270 cal. per mole
Expt. No. 3 (Rabbit)	Normal	E=13,600 cal. per mole
	8 γ per cc. heparin (*in vitro*)	E=12,250 cal. per mole
	12 γ per cc. heparin (*in vitro*)	E=10,550 cal. per mole
	16 γ per cc. heparin (*in vitro*)	E= 7800 cal. per mole
Expt. No. 4 (Pig)	Normal	E=11,150 cal. per mole
	6 γ per cc. heparin (*in vitro*)	E= 9950 cal. per mole
	8 γ per cc. heparin (*in vitro*)	E= 9480 cal. per mole
	12 γ pre cc. heparin (*in vitro*)	E= 8100 cal. per mole

The calculation is illustrated as follows (Expt. No. 3). In Fig. 1 the temperature is plotted against the corresponding K values. If we take any two determinations represented on the curve and substitute the respective T and K values into the above equation, we get the activation energy.

The same calculations were done in the presence of increasing amounts of heparin. The changes in activation energies that were obtained are shown in Table I. From Table I it is clear also that heparin *in vitro* decreases the activation energy of the inactivation of thrombin. Since the characteristic effect of catalysts is that of changing the activation energy of a reaction, heparin can be considered as a catalyst in thrombin inactivation.

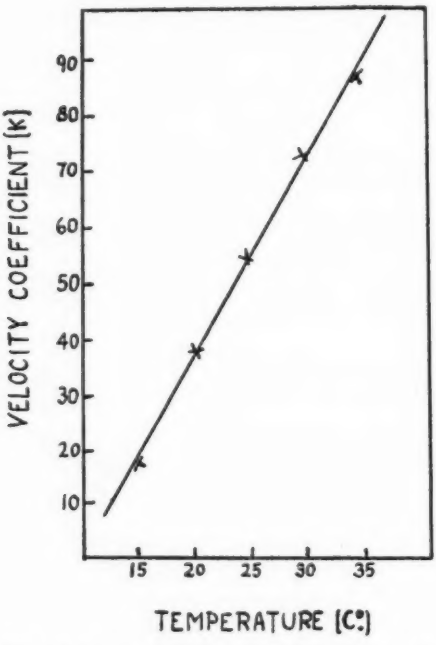

FIG. 1. The effect of temperature on the velocity coefficient of thrombin inactivation.

Acknowledgments

Appreciation is acknowledged to Prof. C. B. Weld for helpful criticism in the preparation of the manuscript.

References

1. ASTRUP, T. and DARLING, S. Naturwissenschaften, 29: 300. 1941.
2. ASTRUP, T. and DARLING, S. Acta Physiol. Scand. 4: 293. 1942.
3. ASTRUP, T. and DARLING, S. Acta Physiol. Scand. 5: 13. 1943.
4. GERENDAS, M. Nature, 157: 837. 1946.
5. GERENDAS, M. Hung. Acta Physiol. 1: 97. 1948.
6. GERENDAS, M. Arch. Biol. Hung. 18: 181. 1948.
7. GERENDAS, M. Orvosi Hetilap, 90: 98. 1949.
8. GETMAN, F. H. and DANIELS, F. Outlines of theoretical chemistry. 6th ed. John Wiley & Sons Inc., New York. 1937.
9. GLAZKO, A. F. and FERGUSON, F. H. J. Gen. Physiol. 24: 169. 1940.
10. GLAZKO, A. F. and FERGUSON, F. H. Am. J. Physiol. 134: 54. 1941.
11. QUICK, A. F. Physiol. Revs. 24: 297. 1944.
12. SCHMIDT, A. Zur Blutlehre. F. C. W. Vogel, Leipzig. 1892.
13. SEEGERS, W. H. Blood coagulation. The Enzymes. Vol. I. Part 2. *Edited by* J. B. SUMNER and K. MYRBACK. Academic Press Inc., New York. 1951.
14. SEEGERS, W. H. and SMITH, H. P. Proc. Soc. Exptl. Biol. Med. 52: 159. 1943.
15. SEEGERS, W. H., WARNER, E. D., BRINKHOUS, K. M., and SMITH, H. P. Science, 96: 300. 1942.
16. VOLKERT, M. Acta Physiol. Scand. 5: 16. 1942.
17. WEST, E. S. Physical chemistry. The MacMillan Company, New York. 1942.

THE ELASTICITY OF THE LARGE ARTERIES IN HYPERTENSION[1]

By J. P. Adamson and J. Doupe

Abstract

Intra-arterial pressures and pulse wave velocities were measured in 18 subjects whose auscultatory diastolic pressures ranged from 45 to 120 mm. Hg. Various methods were used to lower the blood pressure in the hypertensive and to raise it in nonhypertensive subjects so that pulse wave velocities might be compared in all subjects at a common diastolic pressure. The pulse wave velocities were calculated for a diastolic pressure of 80 mm. Hg. No significant differences were found between hypertensive and nonhypertensive subjects. It was concluded that a defect of arterial elasticity as gauged by pulse wave velocity is not a factor in the pathogenesis of hypertension.

Introduction

This investigation of the volume elasticity of the large arteries in hypertensive subjects was undertaken because of the possibility that a disturbance of the pressure reservoir function of these vessels might play a part in the genesis of hypertension. Previous workers, by measuring the pulse wave velocity, which is inversely related to elasticity (3), have shown that elasticity decreases as the pressure increases. They concluded therefore that the decreased elasticity in hypertension was simply the expected consequence of the raised pressure and that there was no primary defect in the large vessels. Nevertheless these previous studies seemed to require confirmation because some (4, 11, 12) were made using the auscultatory method of sphygmomanometry, which has since been shown to be unreliable (8, 9). On the other hand Steele's observations (7), which were related to intra-arterial pressures, were restricted to a short segment of the brachial artery.

The problem was approached in a fashion similar to that of Steele who measured the pulse wave velocity at a standard diastolic pressure of 80 mm. Hg. Steele, however, manipulated the pressure by means of an armlet whereas in this study the general arterial pressure was altered by the use of drugs or other maneuvers.

Subjects and Methods

The subjects were all ambulant without congestive failure and were chosen so that the diastolic pressures in the group would cover a wide range. To help eliminate an age factor, those under 35 years were excluded. Basal auscultatory pressures were measured on several occasions after the subjects had rested for 30 min. in the supine position; the change in sound was used as the criterion of the diastolic pressure. The average values are given in Table I. Certain individuals had hard, irregular, peripheral vessels or calcification of the aorta; these are designated in Table I as arteriosclerotic. Others

[1] *Manuscript received October 9, 1951.*

Contribution from the Department of Physiology and Medical Research of The University of Manitoba, Winnipeg, and from the Winnipeg General Hospital, Winnipeg, Man.

who had a diastolic pressure consistently over 90 mm. Hg. on several occasions and who also had eye ground changes, roentgenographic evidence of left ventricular enlargement, or diminished renal function were said to have essential hypertension. One subject had luetic aortic insufficiency while the remainder, as indicated in the table, were not considered to have a cardiovascular abnormality.

The intra-arterial pressure was measured in the brachial artery by means of the Lilly capacitance manometer (6) and amplifier (10). The recording device was a mirror galvanometer which reflected a beam of light onto photosensitive paper moving at a speed of 125 mm. per second. Four pulse wave tracings were simultaneously recorded on the same photosensitive paper by reflecting the same beam of light from mirrors mounted on Clarke's capsules which, in turn, were connected by thick rubber tubing to glycerine tambours situated over the brachial, subclavian, femoral, and dorsalis pedis arteries. When the tambours were simultaneously activated by a sudden pressure, the time lag between the first and last deflections of the four tracings was less than 4.0 msec.; appropriate corrections were made when analyzing the tracings.

The experiments were performed in a quiet room with the subjects recumbent. After they had rested for at least 45 min., a control record consisting of 12-25 pulse cycles was taken. To alter the blood pressure, one or more of the following procedures was used:

1. The cold pressor test, as described by Hines and Brown (5), except that the foot instead of the hand was immersed in ice water.
2. Exercise of the calf muscles when a cuff around the thigh was inflated to 200 mm. Hg (1).
3. Intravenous epinephrine administered at a rate of 25 μgm. per minute for a period of two-five minutes.
4. Intravenous tetraethyl ammonium chloride, 7 mgm. per kgm. body weight administered in 30 sec.
5. Inhalation of amyl nitrite vapor.
6. Intravenous histamine administered at a rate of 10 μgm. per minute for a period of 3-10 min.

Records were taken as soon as it was thought that the maximum hypotensive or hypertensive effect had occurred.

In all experiments, estimates were made of the pulse wave velocities from subclavian to brachial, subclavian to femoral, and from femoral to dorsalis pedis arteries. To derive pulse wave velocity, the distance traveled by the pulse waves was obtained by applying the formulae of Bazett (2) and the time required at the diastolic pressure was obtained by measuring the interval between the initial upstrokes of the appropriate pulse tracings. The measurement of distances may be expected to give comparative values except in cases of marked arteriosclerosis where tortuosity of vessels may introduce a major error. The pulse wave velocity and blood pressure values recorded

TABLE I

DATA PERTAINING TO SUBJECTS AND PULSE WAVE VELOCITIES

Case No	Sex, age	Average basal auscult. B.P.	Resting int.-art. B.P.	Resting p.w.v. in m./sec.				Diastolic pressure range explored	Average p.w.v. at 80 mm. Hg, m./sec.	Clinical diagnosis	Experimental procedures*
				Subcl. brach.	Subcl. fem.	Fem. d.p.	Average				
1	M/57	180/45	176/34	6.9	6.0	8.3	7.1	31–46	10.2	Aortic regurg.	1, 5, 6
2	M/50	105/60	94/47	9.2	7.2	8.1	8.2	32–68	11.1	Peptic ulcer	1, 2, 3, 5
3	M/54	120/70	89/57	9.2	6.7	9.0	8.3	57–63	9.4	Emphysema	1, 3, 5
4	M/76	135/70	106/46	7.4	10.0	5.4	7.6	36–55	9.4	Emphysema	1, 3, 5
5	F/60	130/72	181/83	8.6	9.8	11.3	9.9	83–102	9.6	Obesity	1, 5
6	M/65	186/80	208/89	9.2	10.1	15.6	11.6	81–97	11.4	Arteriosclerosis	1, 5
7	M/55	160/80	179/75	7.1	6.3	10.8	8.1	65–77	8.5	Chronic nephritis	1, 5
8	M/78	140/88	169/73	10.8	8.8	8.6	9.4	70–89	10.5	Arteriosclerosis	1, 2, 5
9	M/37	145/90	151/88	12.7	10.0	11.4	11.4	47–88	11.0	Hypertension	1, 3, 5
10	F/56	190/100	217/100	10.8	10.7	16.4	12.6	86–136	11.5	Hypertension	1, 4, 5
11	F/45	164/100	180/95	8.0	9.4	12.3	9.9	76–95	9.0	Hypertension	4, 5
12	F/59	160/100	192/92	8.1	11.5	13.9	11.2	70–92	10.4	Hypertension	5,
13	F/49	150/100	198/107	12.3	8.2	16.3	12.3	92–110	10.5	Hypertension	4, 5
14	F/68	200/100	222/94	8.6	9.1	15.7	11.1	86–108	10.8	Hypertension	1, 5
15	M/57	170/105	162/76	9.9	6.7	13.3	10.0	49–90	10.3	Hypertension	1, 5
16	F/52	170/106	194/95	13.2	11.9	12.8	12.6	72–108	11.8	Hypertension	5
17	M/75	225/120	192/79	6.7	10.5	10.2	9.1	69–82	8.6	Hypertension	5, 6
18	M/42	200/120	200/102	10.5	10.0	11.4	10.6	44–102	9.9	Hypertension	4, 5

**Numbers refer to procedures listed in the section on Subjects and Methods.*

represent the mean values of at least 10, and often more, cycles. In each subject these were obtained at two to five pressure levels, the range being shown in Table I.

Results

The results under basal conditions are listed in Table I. Also listed is the average pulse wave velocity at a diastolic pressure of 80 mm. Hg. This figure was obtained for each subject by plotting the average pulse wave velocity against the diastolic blood pressure. Inter- or extrapolation gave the velocity at 80 mm. Hg.

Almost invariably, in each patient with change in blood pressure, the changes in pulse wave velocity were similar in the three parts of the arterial tree examined. In the individual subjects the average pulse wave velocity increased with an increase in diastolic pressure. Table I shows that for the group resting pulse wave velocities were similarly related to pressure.

The relationship between the pulse wave velocity calculated for a diastolic pressure of 80 mm. Hg. and the resting auscultatory diastolic pressure is shown in Table I. It will be seen that there was only a slight tendency for this pulse wave velocity to be elevated in the subjects with high resting diastolic pressures. When the group was divided into those with resting intra-arterial diastolic pressures above and below 80 mm. Hg., the former showed a mean pulse wave velocity of 10.6 meters per second and the latter 9.7 meters per second, and the ranges overlapped considerably.

Discussion

In order to determine whether an intrinsic abnormality of arterial elasticity is associated with hypertension, one must have some measure of the former and some criterion of the severity of the latter. Arterial elasticity is dependent on the characteristics of the vessel, i.e., its cross-sectional area and the composition of its wall, and is also affected by the lateral pressure exerted by the contents. It is therefore necessary to exclude or control the pressure factor. In obtaining the results reported above, an attempt to do so was made by measuring the elasticity, as indicated by the pulse wave velocity, at a pressure of 80 mm. Hg. The procedures adopted to raise or lower the pressure to this level could themselves have affected the characteristics of the vessels. This possibility was controlled so far as was possible by using a diversity of agents.

To assess the degree of hypertension both auscultatory and intra-arterial diastolic pressures have been used. Both show that the resting diastolic pressure is not related to arterial elasticity, as measured by the pulse wave velocity at a diastolic pressure of 80 mm. Hg. Furthermore, as the subjects with the higher resting diastolic pressures showed clinical evidence that the hypertension was having adverse effects, it appears that these complications are also unrelated to arterial elasticity.

These findings substantiate the deductions of Steele (7), who attributed the high pulse velocities found in hypertension to the high internal pressure, and

therefore the conclusion can be drawn that defective arterial elasticity is not a factor in the pathogenesis of diastolic hypertension.

Acknowledgments

The work reported in this paper was performed while J.P.A. was a Research Fellow of the Canadian Life Insurance Officers' Association. The equipment was supplied by Mr. W. McG. Rait of Winnipeg and by the Manitoba Institute for the Advancement of Medical Education and Research.

References

1. ALAM, M. and SMIRK, F. H. J. Physiol. (London) 89: 372. 1937.
2. BAZETT, H. C., COTTON, F. S., LAPLACE, L. B. and, SCOTT, J. C. Am. J. Physiol. 113: 314. 1935.
3. BRAMWELL, J. C. and HILL, A. V. Proc. Roy. Soc. (London), B, 93: 298. 1922.
4. HAYNES, F. W., ELLIS, L. B., and WEISS, S. Am. Heart. J. 11: 385. 1936.
5. HINES, E. A. and BROWN, G. E. Am. Heart J. 11: 1. 1936.
6 PETERSON, L. H., DRIPPS, R. D., and RISMAN, G. C. Am. Heart J. 37: 771. 1949.
7. STEELE, J. M. Am. Heart J. 14: 452. 1937.
8. STEELE, J. M. J. Mt. Sinai Hosp., N.Y. 8: 1042. 1942. *Cited by* BRADLEY, S. E. Am. J. Med. 4: 398. 1948.
9. THOMSON, A. E. and DOUPE, J. Can. J. Research, E, 27: 72. 1949.
10. TOMKINS, H. E. Am. Heart J. 37: 783. 1949.
11. WEISS, S., HAYNES, F. W., and SHORE, R. Am. Heart J. 11: 402. 1936.
12. WIGGERS, C. J. Am. Heart J. 16: 515. 1938.

A MECHANICAL HEART AND LUNG[1]

By Russell A. Waud

Abstract

A practical mechanical heart and lung is described in which glass syringes are used to replace the ventricles. The pistons of the syringes are operated by a special cam rather than a simple crank. Thus the volume changes in the syringes are made to follow those of the mammalian ventricles. The heart rate and stroke output may be varied at will and read at any moment. The artificial lung consists of a large percolator contained in a water bath. Oxygenation of the blood is maintained mainly by passing it through an aggregation of oxygen bubbles rather than by passing the oxygen through the blood. Connections of the apparatus to the animal are such that the blood is withdrawn from the right auricle and replaced into the aorta at its base. A technique has been developed whereby only 20 sec. are required to change from the animal's heart and lungs over to the mechanical apparatus. Thus the organs, including the brain, suffer little hypoxia during the change.

Introduction

A number of perfusion pumps, mechanical hearts, and lungs or oxygenators has been devised (1-14). After experimentation with various methods it is felt that the apparatus and technique to be described will enable the average experimentor to study the peripheral circulation and, at the same time, control or modify at will the rate, stroke output, etc. of the heart. A carefully worked out technique reduces the period of low blood pressure and resulting hypoxia to a minimum and thus preserves the nervous system as evidenced by active eye and other reflexes.

Apparatus

The principle of the artificial heart (Fig. 1, top) to be described here, differs from that of hearts previously constructed; first in that 50 ml. glass syringes, from which the stroke output can be read at any moment, are used to replace the ventricles. Secondly, by means of a specially designed cam the volume changes in the syringes are made to follow the volume changes of the mammalian ventricle as described by Wiggers and Katz (17). In the case of the artificial lung (Fig. 2) to be described, oxygenation of the blood depends mainly upon passing the blood through an aggregation of oxygen bubbles rather than the passage of oxygen through the blood. The apparatus was designed primarily for physiological experimentation and to study the action of drugs on a heartless–lungless preparation. However, it can be used in cardiac surgery by slightly modifying the technique. It is the counterpart of the Starling heart–lung preparation.

An electric motor, as a source of power, is connected through a mechanical device so as to reduce and to vary at will the speed of the shaft to which the

[1] *Manuscript received December 6, 1951.*

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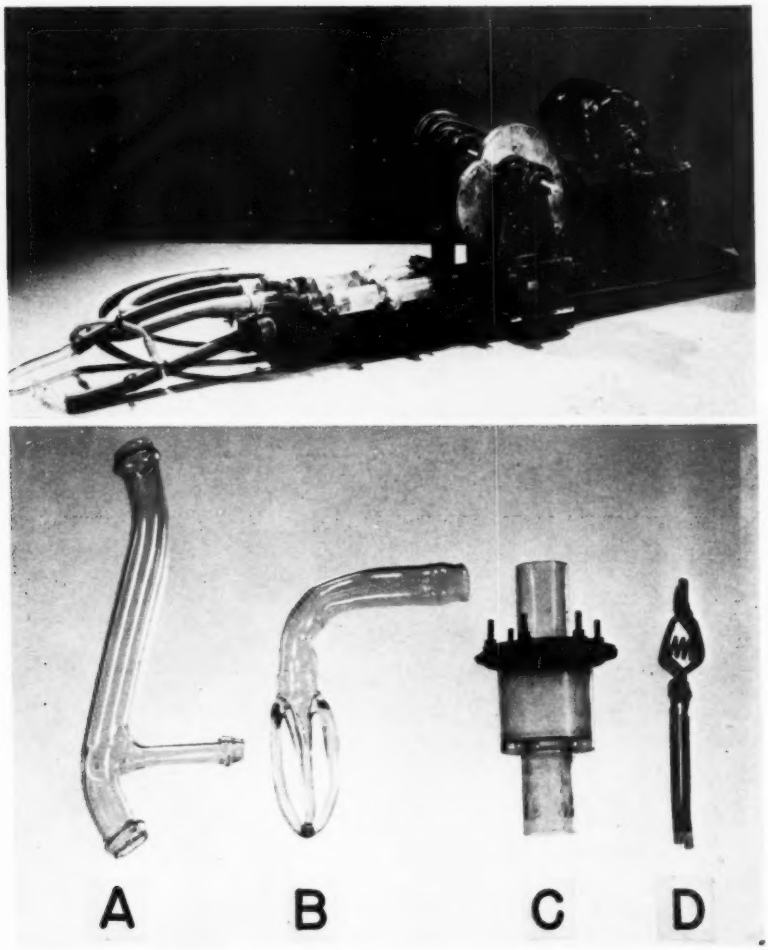

FIG. 1. Top: The mechanical heart with attached valves, special cannulae, and connecting tubing.

Bottom: *A*. The special aortic glass cannula, used to connect the output tube of left syringe with the aorta.

B. The special auricular glass cannula, used to connect the right auricle with the input tube of the right syringe. The ribs prevent collapse of the auricle.

C. A rubber flap valve in its plastic case. One is contained in each tube carrying blood to and from the syringes.

D. A special bulldog on which the jaws have been lengthened. It is used while operating on the appendage of the auricle.

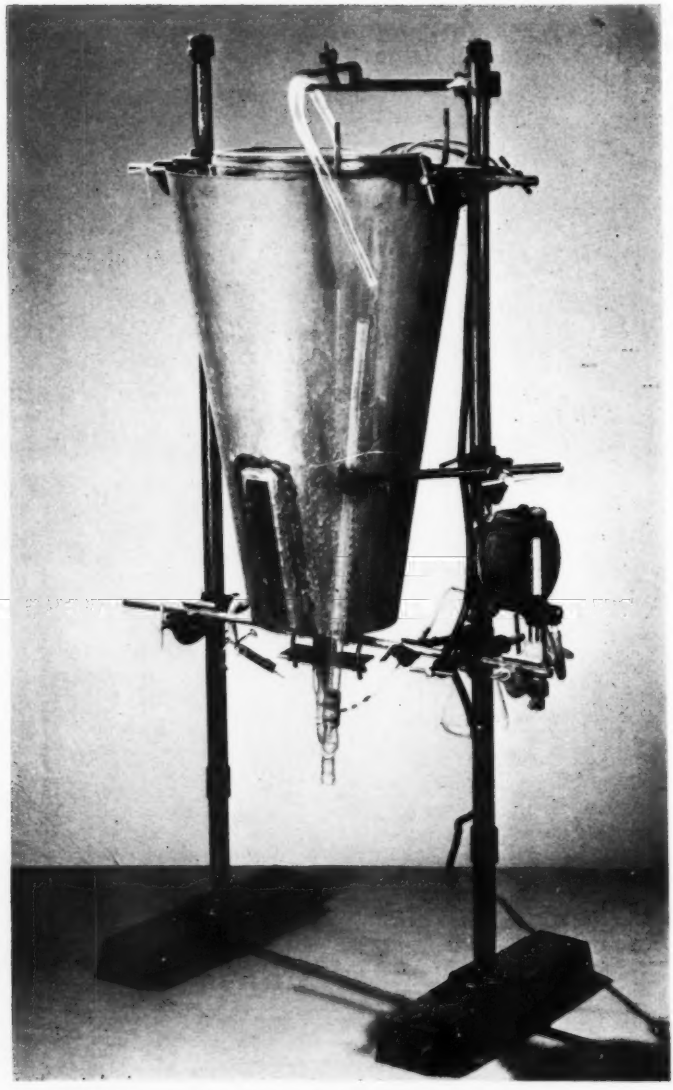

FIG. 2. The mechanical lung with glass percolator inside the water bath.

specially designed cam is attached. The ventricular volume curve was transposed to, and cut out of, the periphery of a circle to construct a cam in such a manner that one whole cardiac cycle occupied the time of a single revolution of the cam. The movements of the cam are transmitted to the plungers of the syringes by means of a lever and two connecting rods. A ball bearing on one end of the lever is made to follow a groove in the cam. In order to vary at will the length of the stroke, and thus the stroke output, the position of the fulcrum on the lever may be varied by means of a thumb screw. The syringes were modified for use in the apparatus by having the ends sawn off and replaced by plastic caps, each cap having two oval tubes of maximum size. The lower tube on the right syringe is connected by means of a rubber tube to the right auricle and the upper tube to the intake of the oxygenator. The lower tube of the left syringe is connected to the return from the oxygenator and the upper tube is connected to the aorta. Specially designed cannulae (Fig. 1, A and B) serve to connect the rubber tubing to the auricle and aorta. A simple flap valve (Fig. 1, C) is inserted in each tube proximal to the end of each syringe. A T-tube is inserted in the tube leading from the right auricle to the right syringe and its side tube is connected by rubber tubing to the aortic cannula. A Hoffman clamp is placed on the connecting tube and closed.

The artificial lung (Fig. 2) is simple and consists mainly of an 8 liter glass percolator, the bottom hole of which is large enough to admit the oxygen and blood return tubes. The percolator is contained in a copper water bath of similar shape and is provided with a drain cock. The main supports of the artificial lung are two $\frac{1}{2}$ in. by 3 ft. iron pipes, each attached to a wooden base by means of a floor flange. The wooden bases are 1 ft. long by $1\frac{1}{2}$ in. thick, and are the width of the floor flange. The bases lie parallel with the animal table and rest on it beside the abdomen of the animal.

The bottom, or neck, of the percolator passes through a rubber stopper in the bottom of the copper bath. The copper bath rests on a brass plate with a large hole in the center, through which the neck of the percolator protrudes. Bolted to the plate and extending from opposite sides are two $\frac{3}{8}$ in. brass rods, which are fastened to the main supports by means of large double clamps. A rubber stopper with one large and one small hole is inserted in the neck of the percolator. A brass plate with a hole in the center, smaller than the stopper, is held against the stopper by means of bolts which hook over the $\frac{3}{8}$ in. brass rods. This serves to keep the rubber stopper in place. Oxygen, after passing through a flowmeter and trap, enters the bottom of the percolator through a small tube passing through the small hole in the rubber stopper. From the tube, it enters the blood through the perforated plate of a Buchner funnel, 5 cm. in diameter. A second and larger glass tube passing through the large hole in the rubber stopper conveys the blood from the percolator to the rubber tubing leading to the left pump. A Y-tube, inserted between the glass tube and the rubber tubing is extended upward. This serves to indicate the blood level in the percolator and to trap any stray bubbles of oxygen. A Hoffman clamp is placed on the rubber tubing leading to the pump. This

clamp is kept closed to the point where the rubber tubing collapses slightly with each stroke of the pump. This is necessary in order to compensate for the ease with which the blood flows from the artificial lung to the left syringe, compared with that from the great veins to the right syringe. Alterations in venous supply affect the output of even a mechanical heart where nonrigid tubes are used.

A large brass ring, passing around the top of the percolator, with $\frac{3}{8}$ in. rods extending from either side, supports the top of the percolator and the copper bath. The rods pass through a notch in the top of the bath, and are attached to the main supports by large double clamps. A plastic receptacle, 7 cm. deep and of such a diameter as to fit into the top of the percolator, receives the blood from the right syringe. The bottom is perforated with small holes of such a design that the blood is distributed evenly over the aggregation of oxygen bubbles in the percolator. A U-shaped glass tube supported by a Bunsen clamp attached to one of the main supports is mounted over the plastic receptacle. Attached to the central end is a cloth bag which would filter out any fibrin. The other end of the U-tube is attached to the output tube of the right syringe.

By means of an immersion heater, the water in the water-jacket is kept at 40°C. or higher, depending on the rectal temperature. Blood is pumped by the right syringe from the right auricle into the top of the percolator, where it passes through the filter into the plastic receptacle and from there drips into the percolator. Thus, oxygenation is accomplished by allowing the blood to pass through the interspaces of the mass of oxygen bubbles produced by bubbling oxygen through the blood. The blood collects at the bottom of the percolator and is pumped by the left syringe into the aorta.

Technique

Dogs weighing 10-20 kgm. are anaesthetized with morphine and ether or other suitable anaesthetic, depending on the nature of the experiment, and arranged for recording blood pressure, etc. The apparatus through which the blood flows, with the exception of the syringes, could be treated with silicone to reduce the danger of clotting. This would be desirable but we have not found it necessary.

The mechanical heart and lung are now brought into position over the animal, and the return tube from the mechanical lung is connected with the intake of the left syringe. One thousand milliliters of whole blood or whole blood and dextran (Intradex) are placed in the percolator and sufficient oxygen is started bubbling through to form an aggregation of oxygen bubbles. After oxygenation, the blood in the artificial lung is allowed to fill the left mechanical heart and the specially designed arterial cannula (Fig. 1, A) by opening a Hoffman clamp which has been previously placed on the return tube. When the system is completely filled, and all air excluded, a stopper is placed in the end of the aortic cannula. The arterial side of the right mechanical heart is now connected with the inlet at the top of the mechanical lung. The

right mechanical heart and connecting tubes fill automatically with blood from the right auricle and the aortic cannula when the mechanical heart is set in motion. The specially designed venous cannula (Fig. 1, B) connects the inlet tube of the right syringe with the right auricle. The chest is now opened by a median incision and the internal mammary arteries tied. After opening the pericardium, a ligature is placed around the aorta close to the heart but not tied. In order to avoid having to separate the aorta from the pulmonary artery, which operation may result in puncture of either of these arteries, the pulmonary artery is included in the ligature. There is an opening between the vessels and the pericardium through which the ligature may be passed. An ordinary Harvard seeker, the shank and handle of which has been curved, makes a useful instrument for this operation. Heparin, 7.5 mgm. per kgm., is now injected into the femoral vein to prevent clot formation. Repeated doses at hourly intervals may be found necessary. The special auricular cannula, which has been tied into the intake tube of the right syringe is now placed in the right auricle. Two fine haemostats are first placed on the angles of the auricular appendage so as to admit the cannula between them. With these haemostats the assistant holds the appendage in an accessible position. A special bulldog (Fig. 1, D) on which the jaws have been lengthened is next placed on the appendage at its junction with the auricle. A ligature is then placed below this. With curved scissors, an opening is made into the appendage between the haemostats. The cannula is now slipped in and tied by the operator. The bulldog is removed as the cannula is inserted. In order to replace the air in the auricular cannula and tubing with blood, the pump is allowed to make several strokes. During this time the Hoffman clamp on the tube connecting the aortic cannula with the T-tube must be opened. The chest opening should be made as wide as possible to facilitate this part of the operation.

The aortic cannula is next inserted. This usually requires less than 20 sec. and is the only time during which the animal's blood pressure is below an effective level. The ventricles are tightly held in the left hand and an opening large enough to admit the aortic cannula is made through the apex of the left ventricle with a cork borer. The cannula is quickly brought to the edge of the opening and immediately, on removal of the stopper by the assistant, it is passed through the ventricle into the aorta and held while the assistant ties the ligature. The cannula is directed upward and to the right. The minute output of the heart is then adjusted by changes in heart rate or stroke output, so as to maintain the normal blood pressure of the animal. The Hoffman clamp on the tube carrying blood from the lung is partly closed so as to compensate for the pressure in the tube compared with that in the large veins of the animal. This is adjusted until the level of the blood in the bottom of the percolator remains constant. Later in the experiment it may be impossible, owing to failure of some of the blood to return from the animal, to maintain this blood level without adding extra blood or blood substitute to the percolator. The oxygen flow is adjusted to maintain a good supply, but not an

overflow of oxygen bubbles in the oxygenator The animal's temperature should be maintained as near normal as possible in order to facilitate passage of oxygen from the blood into the tissues. If the animal is not too deeply anaesthetized eye and other reflexes are present. When whole blood is used, these reflexes remain active for hours.

Discussion

There are two main uses for which the mechanical heart–lung may be employed. One, as a means of obtaining a dry heart in cardiac surgery; the other for animal experimentation, such as in the study of the effects of drugs on animals (at the same time excluding any action on the heart), or in the study of the effects on the peripheral circulation of changes in the heart rate, stroke output, or minute output of the heart. It is for the latter uses that the above described instrument has been primarily developed. The special cam operating the pumps produces in the arterial system the same sequence of pressure and volume changes for which its walls have been developed and to which they are accustomed. When perfused for any length of time, a proper pulsatile blood flow is apparently necessary for the normal function of the vital organs. The use of graduated glass syringes to replace the ventricles, together with facilities for changing the stroke output and heart rate at will, gives complete control and accurate determination of the cardiac output. For the successful operation of glass syringes as pumps they must be connected to the lever which operates them, by two separate connecting rods; also they should be thoroughly cleaned when first put together and kept constantly lubricated with water. Cotton, kept saturated with water and laid across the plungers where they enter the barrel, will keep them in good working condition. The simplest method of connecting a mechanical heart–lung to the animal is to withdraw the venous blood from the venae cavae by means of one or two catheters in the vessels and return it into the aorta or one of its branches. It has been found in this work however, that with this method a significant quantity of blood is always escaping into the right auricle and, as a result, a considerable amount of the circulation is carried on by the animal's own heart. To avoid this, it was found necessary to withdraw the venous blood from a cannula tied in the right auricle and return the oxygenated blood into the aorta where the latter leaves the left ventricle. By this technique the animal's heart is completely eliminated. When the heart was first constructed (15) the valves were of a pinchcock type, operated by cams. This type of valve was found to be difficult to keep properly adjusted. The ordinary flap-valve, when supplied with sufficient tension, has been found to be very satisfactory and not difficult to construct (16).

The use of an artificial lung accomplishes two things. First it obviates termination of the experiment in one to two hours owing to pulmonary oedema, which is so frequently developed when the animal's own lung is used. Secondly, it simplifies the technique of the operation by eliminating the necessity

of cannulating the left auricle and the pulmonary artery. The latter cannulation involves passing a ligature between the aorta and the pulmonary artery. This is a dangerous procedure, quite liable to lead to rupture of one of these vessels.

In the construction of an artificial lung, the degree of oxygenation obtained is proportionate to the surface area of the blood coming in contact with the oxygen. In experiments carried out in this work, it has been found that the aggregation of oxygen bubbles interspaced with blood best satisfies this condition. The blood is separated from the bubbles by gravity and collected at the bottom of the percolator from which it passes to the left pump. The size of the aggregation of bubbles is readily controlled by the rate of flow of oxygen. In order to do this, it may be necessary at times to shut off the oxygen completely.

In the development of the instrument, every effort has been made to simplify it as much as possible and to develop the technique to the point where a preparation using a mechanical heart and lung may be set up in the ordinary experimental laboratory with little fear of failure. It is felt that this has been accomplished. We have used this apparatus on over 100 dogs.

Acknowledgment

This investigation has been made possible by grants from the Medical Division of the National Research Council of Canada.

References

1. BAYLESS, L. E., FEE, A. R., and OGDEN, E. A. J. Physiol. (London), 66: 443. 1928.
2. BJÖRK, V. O. Lancet, 2: 491. 1948.
3. BRULL, L. Science, 111: 277. 1950.
4. CARREL, A. and LINDBERGH, C. The culture of organs. Paul B. Hoeber Inc., New York. 1938.
5 CLARK, L. C. Science, 111: 85. 1950.
6. DALE, H. (SCHUSTER, E. H. J.) Lancet, 1: 982. 1928.
7. DALY, I. and THORPE, W. V. J. Physiol. (London), 79: 199. 1933.
8. DRINKER, K. C., DRINKER, K. R., and LUND, C. C. Am. J. Physiol. 62: 1. 1922.
9. GIBBS, O. S. J. Pharmacol. Exptl. Therap. 38: 197. 1930.
10. HOOKER, D. R. Am. J. Physiol. 38: 200. 1915.
11. JONGBLOED, J. Surg. Gynecol. Obstet. 89: 684. 1949.
12. RICHARDS, A. N. and DRINKER, C. K. J. Pharmacol. Exptl. Therap. 7: 467. 1915.
13. SCHRODER, W. Arch. exptl. Path. Pharmakol. 15: 364. 1882.
14. STOKES, T. L. and GIBBON, J. H. Surg. Gynecol. Obstet. 91: 138. 1950.
15. WAUD, R. A., GOWDEY, C., and LOYNES, J. J. Pharmacol. Exptl. Therap. 98: 34. 1950.
16. WAUD, R. A., LOTZ, F., and BOYD, J. Rev. can. biol. 10: 93. 1951.
17. WIGGERS, C. J. and KATZ, L. N. Am. J. Physiol. 58: 439. 1922.

NICOTINAMIDE HAEMOCHROME* AS A DERIVATIVE FOR THE COLORIMETRIC DETERMINATION OF THE TOTAL HAEM PIGMENTS IN BLOOD[1]

By F. D. White, G. E. Delory, K. A. Christie, and E. A. Dudley

Abstract

When nicotinamide is added to a solution of blood in $N/10$ sodium hydroxide, and the mixture reduced with sodium dithionite, a haemochrome is formed with two well marked absorption bands. This observation forms the basis of a simple colorimetric method for the determination of the total haem pigments in blood. This method, utilizing the innocuous nicotinamide in place of either cyanide or pyridine, has been shown to be precise and to give results whose accuracy has been confirmed by iron analysis.

Introduction

Since 1878 when Gowers (6) measured the amount of haemoglobin in blood by comparison with a picrocarmine standard, many attempts have been made to evolve a simple colorimetric procedure by which the haem pigment content of blood could be determined with a fair degree of accuracy. These methods generally were based upon a preliminary treatment of the blood sample to form a stable haem derivative which could then be compared against a standard treated similarly. The standard used was either blood whose haem content had been determined by oxygen capacity or iron analysis, or crystalline haemin of known iron content, although in some cases an artificial standard, calibrated in terms of haemoglobin, has been employed. Of these procedures the cyanmethaemoglobin method of Stadie (14), the cyanhaematin method of King and Gilchrist (9), and the pyridine haemochrome method of Roets (13) have all been shown by competent investigators to give reasonably accurate results (3, 10, 12). The pyridine haemochrome method has not been widely adopted because of the unpleasant odor of pyridine, and the cyanhaematin method, although convenient and precise, has met with prejudice in some quarters because of the use of cyanide, although as King and Gilchrist have pointed out no fatalities have been reported.

In the course of a study of haemochrome formation in this laboratory, it was noticed that nicotinamide behaved like pyridine in readily forming a haemochrome with well marked absorption bands. When a sample of blood was treated by the procedure for the formation of pyridine haemochrome, but with the pyridine replaced by a solution of nicotinamide, the resulting red solution viewed in the Beck-Hartridge Reversion Spectroscope showed

[1] *Manuscript received December 3, 1951.*

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**Note on terminology: There is still no agreement on the terminology used to designate the various haem pigments. We have therefore followed the suggestions of Lemberg and Legge* (11) *and throughout this paper the word "haemochrome" has been used instead of "haemochromogen" to denote the compound formed by the conjugation of ferroporphyrin with base.*

absorption bands at wave lengths of 563.7 and 529.8 mμ. A solution of nicotinamide haemochrome prepared from haemin in an exactly similar manner showed corresponding bands at 563.5 and 530.1 mμ respectively. As confirmation, the respective absorption curves were determined with the Beckman DU Spectrophotometer by plotting the optical density against the wave length. Both curves showed peaks at 564 and 530 mμ (see Fig. 1). Although

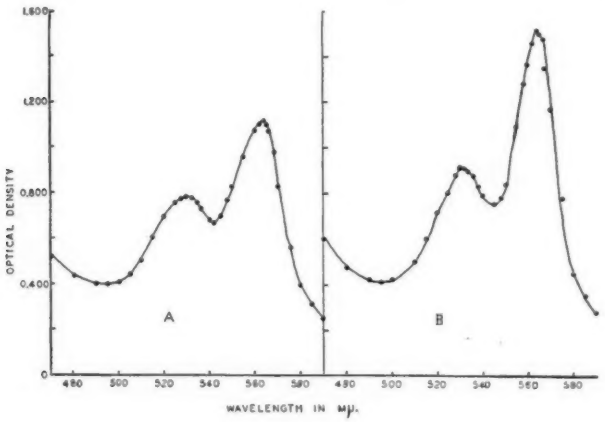


FIG. 1. Absorption curves of nicotinamide haemochrome: A, prepared from the haemoglobin of blood; B, prepared from haemin.

the curves were not identical, since the two solutions were of different iron content, their similarity in shape indicated the possibility of a constant relationship between the haemochrome formed and the haem pigments from which it was derived. Before evaluating the color of the haemochrome solution in terms of the iron content of the blood sample, it was desirable to find the optimum conditions for maximum color development. This settled, the specific extinction coefficient of the haemochrome was determined, i.e. the ratio of the optical density at a chosen wave length to the iron content in mgm. per 100 ml. of the same solution, and, this proving to be a constant value, a method was evolved for the determination of the total haem pigments in blood.

Experimental

Rate of Color Development

Preliminary observations having shown that the nicotinamide haemochrome, like the corresponding pyridine compound (Collier (3)), did not achieve full color development immediately, the following experiment was carried out. To a test tube containing 20 ml. of $N/10$ sodium hydroxide was added 0.1 ml. of blood, followed by a knife-point of sodium dithionite ($Na_2S_2O_4$) and 1 ml. of a 40% solution of nicotinamide, the solution mixed by rotation, and the optical density of the resultant color read in a Beckman

Spectrophotometer. This reading was repeated at three minute intervals until constant. The results showed progressive color development for 30 min. This experiment was repeated many times and invariably it took 30 min. before the optical density attained its maximum value. In a second experiment carried out in exactly the same way, but without the addition of nicotinamide, approximately 20 min. was required for full color development, indicating that the time lag was apparently due mainly to the reduction of the ferric iron to the ferrous state. Attempts to shorten this period by immersing the tube in boiling water, or water at 60°C., and adding the nicotinamide and dithionite together or separately, before and after heating, were unsuccessful. Heating certainly increased the rate of color development, but the final color was in every case less intense than with development at room temperature. It was evident that 30 min. at room temperature was the minimum time required for maximum color development and accordingly this time period was adhered to in all subsequent work.

Determination of the Specific Extinction Coefficient

A 15 ml. sample of oxalated whole blood was haemolyzed with a few drops of ether, centrifuged, and the clear haemolyzate used for both the haemochrome formation and iron analysis. For the former, duplicate 1 ml. samples were pipetted into 250 ml. volumetric flasks containing about 150 ml. $N/10$ sodium hydroxide solution, and the flasks made up to the mark with the sodium hydroxide solution. After thorough mixing, aliquots of 25 ml. were pipetted from each flask into large test tubes and to each was then added 1 ml. of a 40% aqueous solution of nicotinamide, followed immediately by a knife-point of sodium dithionite. The tubes were then rotated until all the dithionite had dissolved and set aside for 30 min. to allow maximum color development. The optical densities were read in two different instruments, the Beckman DU Spectrophotometer with a slit width of 0.05 mm. at a wave length of 545 mμ, and the Hilger Biochem Absorptiometer using a Chance green filter.

Iron analyses were carried out on duplicate 5 ml. samples of the haemolyzate by the titanous chloride method of Delory (4) and the specific extinction coefficient in terms of 1 mgm. iron per 100 ml. of the final colored solution calculated for each of the duplicates.

Over a period of time, 15 different samples of blood were treated in the above manner and the results obtained are shown in Table I. These results show that the specific extinction coefficient is a constant reproducible value, capable of being determined with a reasonable degree of precision either with a spectrophotometer using a specific wave length, or with a photoelectric colorimeter using a filter. With the latter instrument the standard deviation from the mean was 0.047 and the coefficient of variation 1.61%, while with the more precise spectrophotometer the respective values were correspondingly less. Utilizing this specific extinction coefficient the following method was adopted for the determination of the total haem pigments in blood.

TABLE I

THE SPECIFIC EXTINCTION COEFFICIENT (E_s) FOR NICOTINAMIDE HAEMOCHROME AS DETERMINED BY MEANS OF THE BECKMAN DU SPECTROPHOTOMETER AT A WAVE LENGTH OF 545 mμ AND THE HILGER ABSORPTIOMETER USING A CHANCE GREEN FILTER

Blood samples				Beckman		Hilger	
No.	Fe content (by anal.) mgm./ 100 ml.	Hb. content (calc.) gm./100 ml.	Fe in diluted sample μgm./ 100 ml.	Opt. dens.	E_s	Opt. dens.	E_s
1	22.5	6.6	86.5	0.264	3.05	0.257	2.97
2	19.8	5.8	76.1	0.235	3.09	0.215	2.83
3	27.0	8.0	103.8	0.317	3.05	0.302	2.91
4	35.3	10.4	135.8	0.417	3.07	0.401	2.95
5	35.5	10.4	136.5	0.420	3.08	0.391	2.87
6	36.6	10.8	140.8	0.430	3.05	0.411	2.92
7	39.7	11.7	152.7	0.470	3.08	0.440	2.88
8	41.1	12.1	158.1	0.485	3.07	0.461	2.92
9	41.3	12.2	158.8	0.477	3.00	0.470	2.96
10	42.9	12.6	164.6	0.505	3.07	0.473	2.87
11	42.9	12.6	165.0	0.500	3.03	0.482	2.92
12	46.4	13.6	174.6	0.540	3.09	0.511	2.93
13	46.9	13.8	180.4	0.556	3.08	0.542	3.00
14	50.5	14.8	194.2	0.590	3.04	0.560	2.88
15	51.9	15.3	199.6	0.595	2.98	0.570	2.86
Mean value					3.055		2.91
Standard deviation					0.032		0.047
Coefficient of variation					1.05%		1.61%

Method

Pipette 0.5 ml. of oxalated whole blood into a 100 ml. volumetric flask containing about 80 ml. of $N/10$ sodium hydroxide, make up to the mark with more sodium hydroxide and mix. From this solution pipette 20 ml. into a large test tube, add 1 ml. of a 40% solution of nicotinamide, followed by a knife-point (about 50 mgm.) of sodium dithionite. Rotate the tube until the dithionite is dissolved, set aside for 30 min., and then read the optical density in a photoelectric colorimeter.

Micromethod

Pipette 0.05 ml. of blood into 10 ml. of $N/10$ sodium hydroxide, add 0.5 ml. of the nicotinamide solution followed by a knife-point of dithionite. Read as before in the photoelectric colorimeter, after allowing the mixture to stand for 30 min.

Calculation

Taking the iron content of haemoglobin as 0.34% (Bernhart and Skeggs (1)) the amount of haem pigment in 100 ml. of blood is given by the equation:

$$\text{gm. Hb per 100 ml. blood} = \frac{D}{E_s} \times \frac{100}{0.34} \times \frac{1}{1000} \times \text{dilution.}$$

Where D is the optical density, and the dilution, which is the ratio of the volume of final colored solution to the volume of blood contained in it, is 210 for the macro- and 211 for the micromethod.

Using a Hilger Absorptiometer with a Chance green filter the E_s was 2.91, and gm. Hb per 100 ml. blood was therefore

$$D \times \frac{61.8}{2.91} \text{ (macro) or } D \times \frac{62.1}{2.91} \text{ (micro).}$$

Accuracy of the Method

Thirty-one samples of blood were analyzed for total haem pigment by this method and the results were compared against determinations carried out on the same sample by either the cyanhaematin method or by direct iron analysis, and in a few cases by both methods. The results are shown in Table II.

TABLE II

THE TOTAL HAEM PIGMENT CONTENT OF BLOOD AS DETERMINED BY (*A*) THE NICOTINAMIDE HAEMOCHROME METHOD, (*B*) THE CYANHAEMATIN METHOD, AND (*C*) IRON ANALYSIS

Blood sample	Hb content (gm./100 ml. blood)			Ratio	
	Nicotinamide method (*A*)	Cyanhaematin method (*B*)	Iron analysis (*C*)	*A*/*B*	*A*/*C*
1	15.4	15.9	—	0.969	—
2	15.0	15.0	—	1.000	—
3	14.9	15.0	—	0.993	—
4	15.9	15.7	—	1.013	—
5	15.6	16.0	—	0.975	—
6	16.3	16.5	—	0.988	—
7	10.0	9.9	—	1.010	—
8	15.7	16.0	—	0.981	—
9	16.3	16.6	—	0.982	—
10	14.3	14.4	—	0.993	—
11	14.9	15.0	—	0.993	—
12	14.2	14.5	—	0.980	—
13	13.4	13.6	—	0.985	—
14	13.6	13.5	—	1.007	—
15	9.0	8.4	—	1.071	—
16	13.2	13.0	—	1.015	—
17	7.9	7.7	8.0	1.026	0.987
18	12.1	12.3	12.1	0.984	1.000
19	12.6	12.6	12.6	1.000	1.000
20	13.5	13.6	13.6	0.993	0.993
21	14.2	13.4	13.8	1.060	1.029
22	14.7	15.0	14.8	0.980	0.993
23	11.5	—	11.4	—	1.008
24	16.6	—	16.9	—	0.982
25	13.9	—	13.4	—	1.037
26	16.1	—	16.4	—	0.982
27	12.7	—	12.7	—	1.000
28	12.8	—	12.9	—	0.992
29	10.1	—	9.9	—	1.020
30	13.2	—	13.1	—	1.007
31	5.1	—	4.9	—	1.041
Mean value				1.000	1.005
Standard deviation				0.0256	0.0188
Coefficient of variation				2.56%	1.87%

Determination of the iron content was carried out on only about one-half of the samples analyzed since it is a time-consuming process, while the cyanhaematin method which was chosen as one of the most accurate procedures in general use was applied to 22 of the 31 samples. The ratio of the results obtained by the nicotinamide method to those by iron analysis gave a mean value of 1.005 with a coefficient of variation of 1.87%, while the mean value of the ratio, nicotinamide to cyanhaematin, was 1.000 with a coefficient of variation of 2.56%.

Discussion

Our results indicate that nicotinamide haemochrome can be used for the determination of the haem pigments of blood, and a method based on the formation of this derivative has yielded results which, on the basis of iron analysis, are of reasonable accuracy, and which compare favorably with those obtained by the cyanhaematin method. The method has the advantage that neither cyanide nor pyridine are required as reagents. It has however the disadvantage that the specific extinction coefficient must first be determined for each individual photoelectric colorimeter employed. This could be obviated by using a standard solution whose color bore a fixed relationship to a definite haem pigment concentration.

The first substance to be studied was a solution of haemin since this compound can be obtained in a pure crystalline condition. Clegg and King (2) recommended for their alkaline haematin method, a standard solution of haemin in $N/10$ sodium hydroxide of such a strength that the color intensity was equivalent to that of blood of known haemoglobin content treated similarly.

Although the haemin standards used by King were undoubtedly stable for several months, as confirmed by one of us (G.E.D.), not all solutions of haemin in sodium hydroxide show the same degree of stability (5). Horecker (7) reported that sodium hydroxide as the solvent could be replaced by the Sorensen borate buffer of pH 9.4 to give a haemin solution of uniform stability, and King (8) agreed that this reagent improved the stability of his standard solution. An investigation of this solution as a standard for the nicotinamide haemochrome however, resulted in the interesting observation that the presence of borate caused a change towards the violet in the position of the absorption bands, possibly due to a displacement of the nicotinamide by borate ions.

Since the use of haemin in sodium hydroxide solution has been criticized on the grounds of instability, and borate buffer has proved unsuitable for nicotinamide haemochrome formation, it appears that haemin is unlikely to provide a satisfactory standard for this method and other haem derivatives are now being investigated in this laboratory. While a stable and reproducible standard would be desirable from the standpoint of convenience the method as it stands is capable of estimating the total haem pigments in blood with ease and precision.

Acknowledgments

This work was carried out during the tenure of a grant from the Division of Medical Research, National Research Council of Canada, for which grateful acknowledgment is made.

References

1. BERNHART, F. W. and SKEGGS, L. J. Biol. Chem. 147: 19. 1943.
2. CLEGG, J. W. and KING, E. J. Brit. Med. J. II: 329. 1942.
3. COLLIER, H. B. Can. Med. Assoc. J. 50: 550. 1944.
4. DELORY, G. E. Analyst, 68: 5. 1943.
5. GIBSON, Q. H. and HARRISON, D. C. Biochem. J. 39: 490. 1945.
6. GOWERS, W. R. Lancet, II: 882. 1878.
7. HORECKER, B. L. J. Lab. Clin. Med. 31: 589. 1946.
8. KING, E. J. Brit. Med. J. II: 349. 1947.
9. KING, E. J. and GILCHRIST, M. Lancet, II: 201. 1947.
10. KING, E. J., GILCHRIST, M., WOOTTON, I. D. P., DONALDSON, R., SISSON, R. B., MACFARLANE, R. G., JOPE, H. M., O'BRIEN, J. R. P., PETERSON, J. M., and STRANGEWAYS, D. H. Lancet, II: 563. 1948.
11. LEMBERG, R. and LEGGE, J. W. Haematin compounds and bile pigments. Interscience Publishers, Inc., New York. 1949.
12. RIMINGTON, C. Brit. Med. J. I: 177. 1942.
13. ROETS, G. C. S. Onderstepoort J. Vet. Sci. Animal Ind. 14: 451. 1940. (*Quoted by* Rimington (12).)
14. STADIE, W. C. J. Biol. Chem. 41: 237. 1920.

THE *IN VITRO* INCORPORATION OF RADIOACTIVE PHOSPHATE INTO RABBIT RETICULOCYTES[1]

By T. D. Lovegrove, H. A. DeLuca, and R. J. Rossiter

Abstract

Suspensions of rabbit red blood cells containing a high percentage of reticulocytes were obtained by the method of successive daily bleedings. When radioactive phosphorus in the form of inorganic phosphate was added *in vitro* to such suspensions, the cells took up the labeled phosphate more rapidly than red cell suspensions not containing a high percentage of reticulocytes. The more rapid incorporation of radioactive phosphate into the cells of a rabbit subjected to daily bleedings was not due to the accompanying lipaemia. When Krebs–Ringer – phosphate was substituted for plasma as the suspending medium, the rate of penetration of labeled phosphate into mature red cells or mixtures of red cells and reticulocytes was slower, but when Krebs–Ringer – bicarbonate was substituted the penetration was more rapid. The rate of uptake was decreased by the presence of cyanide (0.005 *M*) in the suspending medium.

Introduction

It is well known that red blood cells take up radioactive phosphate *in vitro* (See (7) for references). Since this uptake is inhibited by a number of enzyme inhibitors (4, 6, 9), it is presumably dependent upon the metabolic activity of the cells. Recent work from this laboratory has shown that reticulocytes, which have a higher rate of metabolism than mature red cells, also contain a higher concentration of nucleic acid, chiefly pentosenucleic acid (2). It seemed of interest, therefore, to determine whether reticulocytes were more active in taking up radioactive phosphate.

Methods

Animals

The rabbits used were fully grown. No attempt was made to control the age, sex, weight, or the dietary conditions of the animals.

Reticulocytes

A reticulocytosis of 20 to 50% was produced by successive daily bleedings. From 50 to 60 ml. blood was withdrawn from the marginal ear vein each day for three to six days.

Experimental Procedure

Blood (10 ml.) was withdrawn from the marginal ear vein using heparin as an anticoagulant. The blood was centrifuged at 2400 r.p.m. for 15 min. The upper portion of the plasma layer was drawn off and kept in a clean test tube. The remainder of the plasma, the buffy-coat layer, and the upper portion of the red cell layer were then aspirated off and discarded. The lower portion of the red cell layer, not contaminated with white cells, was used for the experiment.

[1] *Manuscript received December 12, 1951.*

Contribution from the Department of Biochemistry, University of Western Ontario, London, Ont. The work was supported by grants from the National Cancer Institute and the National Research Council of Canada.

In a typical experiment 3 ml. of packed red cells and 3 ml. of plasma were added to a small conical flask. To this was added 50 μliters of isotonic saline containing 0.1 μc. P^{32} in the form of sodium dihydrogen phosphate with negligible carrier phosphate. After mixing, 1 ml. of the suspension was withdrawn for the determination of (*a*) the number of counts in 100 μliters of suspension, (*b*) the haematocrit, and (*c*) the reticulocyte count, done on smears supravitally stained with brilliant cresyl blue and counterstained with Wright's stain. Some of the suspensions contained a significant number of large basophilic cells. These were included in the reticulocyte count.

The flask containing the remainder of the suspension was shaken in a water bath at 37°C. After 15, 30, and 60 min., samples of 0.5 ml. were withdrawn. These were spun at 3000 r.p.m. for 15 min. Duplicate samples of the plasma (100 μliters) were pipetted into aluminum planchets and dried under an infrared lamp. The number of counts was determined with a Geiger–Mueller counter. The 100 μliter sample of the whole suspension taken at the beginning of the experiment was treated the same way. Since the number of counts in 100 μliters of the whole suspension did not differ significantly from those obtained when the same quantity of P^{32} was added to distilled water or Krebs–Ringer buffered with either phosphate or bicarbonate, a distilled water control was used in some experiments.

In some experiments the plasma was replaced by Krebs–Ringer – phosphate or Krebs–Ringer – bicarbonate, prepared as described by Umbreit, Burris, and Stauffer (11). The experiments with Krebs–Ringer – bicarbonate were carried out in Warburg flasks previously equilibrated with a 5% carbon dioxide – 95% oxygen gas mixture.

Calculations

The percentage of the added P^{32} that had entered the red cells at a given time interval was calculated as follows:

$$\text{Percentage of added } P^{32} \text{ in cells} = 100 - \frac{C_p}{C_s}(100 - Ht),$$

where C_p = Number of counts in 100 μliters of plasma at the given time interval.

C_s = Number of counts in 100 μliters of whole suspension.

Ht = Haematocrit.

Throughout this work the amount of P^{32} added was 0.1 μc. in 50 μliters saline for each 3 ml. plasma. However, a fourfold change in the P^{32} concentration did not significantly affect the results.

Results

Normal Red Cells

Fig. 1 shows the penetration of P^{32} into red cells from two rabbits before bleeding. The shape of the curves is typical. After about six hours there was no further uptake of P^{32}. As found by Pertzoff and Gemmill (9) for human

blood, the percentage of the added P^{32} taken up by the cells in six hours varied greatly from animal to animal, and even with successive experiments on the same animal. The percentage of the added P^{32} that had entered the cells after six hours was normally between 65 and 85%.

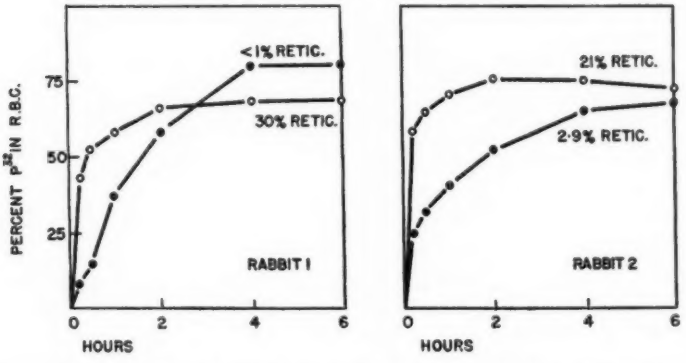


FIG. 1. Penetration of radioactive phosphate into the red cells of the rabbit. ● – ● – ●, before bleeding; O – O – O, after bleeding six times (Rabbit No. 1) and four times (Rabbit No.2). Reticulocyte count as shown.

Red Cells with Reticulocytes

Fig. 1 also shows the penetration of P^{32} into red cells from the same two rabbits after the reticulocyte count had been increased by a number of daily bleedings. In both instances the P^{32} passed more rapidly into the cells containing a high percentage of reticulocytes, although by six hours the percentage of the labeled phosphate that had entered the cells was similar to that observed for the same rabbit before the bleeding. The differences reported for the percentage incorporation after six hours were not significant. Differences of the same order were obtained in successive experiments on animals not subjected to the bleedings.

The curve of P^{32} uptake obtained after the bleedings differed most significantly from the prebleeding curve during the zero to one hour period. Fig. 2 shows this portion of the curve for two more rabbits subjected to daily bleedings. Again there was a much more rapid uptake by the suspensions containing the high percentage of reticulated cells.

Table I shows the uptake of P^{32} by the red cells of 10 normal rabbits and the uptake by the cells of the same rabbits at the height of the reticulocyte response caused by the bleeding. In each instance the percentage of the added P^{32} that had entered the cells at the end of one hour was greater at the time of the reticulocytosis. Table I also gives an indication of the differences in the rate of uptake of P^{32} found for different animals. It can be seen that there was no direct correlation between the extent of the reticulocytosis and the rate at which P^{32} entered the cells. The results of the experiments reported in Fig. 2 suggest that for any given animal some degree of correlation might

exist. This correlation was apparent only if the reticulocyte count was less than 15%. For higher reticulocyte counts there was, in general, no obvious relation between the number of reticulated cells and the P^{32} uptake.

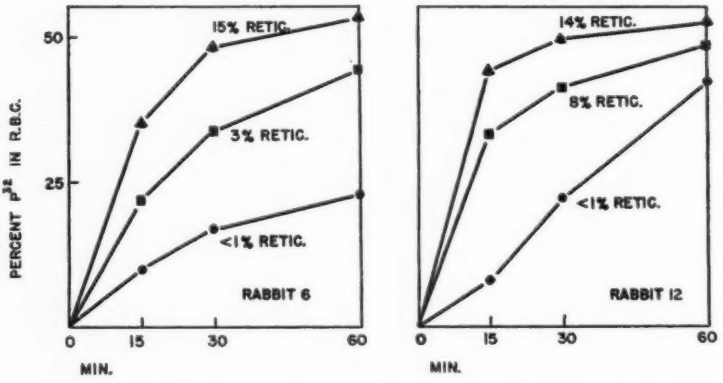


FIG. 2. Penetration of radioactive phosphate into the red cells of the rabbit. ● – ● – ●, before bleeding; ■ – ■ – ■, after bleeding twice (Rabbit No. 6) and four times (Rabbit No. 12); ▲ – ▲ – ▲, after bleeding three times (Rabbit No. 6) and five times (Rabbit No. 12). Reticulocyte count as shown.

TABLE I

PENETRATION OF RADIOACTIVE PHOSPHATE INTO THE RED BLOOD CELLS OF RABBITS BEFORE AND AFTER DAILY BLEEDINGS. SUSPENDING MEDIUM, PLASMA; TIME, ONE HOUR; TEMPERATURE, 37°C.

Rabbit No.	Before bleeding		After bleeding		
	Reticulocyte count, %	Percentage of added P^{32} in cells	Number of bleedings	Reticulocyte count, %	Percentage of added P^{32} in cells
1	<1	35	6	30	59
2	2	41	3	12	70
3	2	26	2	8	50
6	<1	24	5	52	51
7	<1	33	6	86	45
8	2	34	3	34	39
9	<1	44	3	39	51
10	<1	36	4	18	54
11	<1	53	2	11	75
12	<1	42	5	14	51

Possible Effect of Lipaemia

Pertzoff and Gemmill (9) noticed a rapid penetration of P^{32} into the red cells of a patient with lipaemic plasma. Since the plasma of rabbits subjected to daily bleedings rapidly became milky, this aspect of the problem was studied further.

Table II shows that after a number of daily bleedings there was, in addition to the increase of the reticulocyte count, an increase in the optical density of

TABLE II

RETICULOCYTE COUNT, OPTICAL DENSITY OF PLASMA, AND CONCENTRATION OF LIPIDS IN PLASMA OF RABBITS BEFORE AND AFTER DAILY BLEEDINGS

	Rabbit No. 4		Rabbit No. 5	
	Before bleeding	After bleeding, 4 days	Before bleeding	After bleeding, 5 days
Reticulocyte count, %	1.1	33	<1	54
Optical density, at 700 mμ	0.082	0.326	0.088	0.550
Optical density, at 480 mμ	0.26	1.26	0.31	1.29
Plasma cholesterol, mgm./100 ml.	31	144	49	154
Plasma phospholipid, mgm./100 ml.	88	325	94	342
Plasma total fatty acid, mgm./100 ml.	84	1015	—	—

suitably diluted samples of the plasma. The plasma was milky and there was an increase in the concentration of cholesterol, total phospholipid, and total fatty acids. It was, therefore, of interest to determine whether the increased penetration of P^{32} into the cells of the rabbits subjected to the daily bleedings was due to the large number of reticulated cells in the suspensions, or whether it was due to the fact that the cells were suspended in lipaemic plasma.

Table III shows the results of three experiments in which the cells and the plasma of a normal and a lipaemic rabbit were separated. The cells from the

TABLE III

EFFECT OF LIPAEMIA ON THE PENETRATION OF RADIOACTIVE PHOSPHATE INTO THE RED BLOOD CELLS OF THE RABBIT. FIGURES REPRESENT THE PERCENTAGE OF THE ADDED P^{32} THAT HAD ENTERED THE CELLS AFTER ONE HOUR AT 37°C.

	Expt. 1 Rabbit *A*, 43% retic. Rabbit *B*, <1% retic.	Expt. 2 Rabbit *A*, 35% retic. Rabbit *B*, 1.3% retic.	Expt. 3 Rabbit *A*, 31% retic. Rabbit *B*, <1% retic.
High reticulocyte cells (Rabbit *A*) with lipaemic plasma (Rabbit *A*)	53	61	51
High reticulocyte cells (Rabbit *A*) with clear plasma (Rabbit *B*)	61	59	44
Low reticulocyte cells (Rabbit *B*) with lipaemic plasma (Rabbit *A*)	32	49	31
Low reticulocyte cells (Rabbit *B*) with clear plasma (Rabbit *A*)	35	45	32

lipaemic rabbit, which contained a high percentage of reticulocytes, were then incubated with either the lipaemic plasma or the clear plasma from the normal animal. Similarly the cells from the normal rabbit were incubated with either the lipaemic or the clear plasma. In only one of the three experiments with the cells containing the reticulocytes (Expt. 3) and in none of the three experiments with the normal cells, was there a significant increase in the rate of P^{32}

incorporation when the cells were suspended in the lipaemic plasma. On the other hand, in all three experiments there was the characteristic increase in P^{32} uptake when the cell suspensions with the high reticulocyte count were compared with those with the low reticulocyte count, regardless of the degree of lipaemia of the plasma in which they were suspended.

Nature of the Suspending Medium

In the experiments so far reported, plasma was the suspending medium. Table IV shows the effect of substituting Krebs–Ringer for the plasma. In

TABLE IV

EFFECT OF SUSPENDING MEDIUM ON THE PENETRATION OF RADIOACTIVE PHOSPHATE INTO RED BLOOD CELLS OF THE RABBIT. FIGURES REPRESENT THE PERCENTAGE OF THE ADDED P^{32} THAT HAD ENTERED THE CELLS AFTER ONE HOUR AT 37°C.

Reticulocyte count, %	Suspending medium		
	Plasma	Krebs–Ringer – phosphate	Krebs–Ringer – bicarbonate
<1	23	24	
1.6	34	19	
13	46	39	
17	45	34	
17	49	32	
< 1	40		56
2	34		36
30	63		75

four out of five experiments in which the Krebs–Ringer was buffered with phosphate, the P^{32} entered the cells at a slower rate than when the cells were suspended in plasma. On the other hand, where the Krebs–Ringer was buffered with bicarbonate, the P^{32} entered the cells more rapidly. These effects of the suspending medium did not appear to be related to the degree of the reticulocytosis.

TABLE V

EFFECT OF CYANIDE ON THE PENETRATION OF RADIOACTIVE PHOSPHATE INTO THE RED BLOOD CELLS OF THE RABBIT. FIGURES REPRESENT THE PERCENTAGE OF THE ADDED P^{32} THAT HAD ENTERED THE CELLS AFTER ONE HOUR AT 37°C. SUSPENDING MEDIUM, PLASMA

Reticulocyte count, %	No cyanide	Cyanide, 0.005 *M*
<1	56	47
<1	51	49
27	48	40

Effect of Cyanide

Table V shows that the rate of incorporation of P^{32} into the cells was decreased by the addition of cyanide (0.005 *M*) to the suspending medium.

The observations on normal cells confirm those of Hahn and Hevesy (6). The table also shows that there was a similar cyanide inhibition when the red cell suspension contained a high percentage of reticulated cells.

Discussion

The increased uptake of P^{32} by cell suspensions containing a high percentage of reticulocytes is probably related to the increased metabolic activity of such suspensions. They contain a high concentration of acid-soluble and nucleic acid phosphorus and have a high rate of metabolism (2). It is therefore reasonable to suppose that inorganic phosphate is more rapidly esterified, a condition that would favor the faster penetration of inorganic phosphate into the cell. It has been reported, however, that the mature red cells of the hen and frog take up P^{32} less rapidly than do the red cells of man and the rabbit (6). This finding is difficult to explain on the above hypothesis, for these cells, which are nucleated, contain a higher concentration of nucleic acid and have a higher rate of metabolism. The increased metabolic activity of rabbit red cell suspensions containing a high percentage of reticulocytes is also evidenced by their more rapid *in vitro* incorporation of either glycine labeled with N^{15} (8) or labeled leucine (1).

As pointed out by Hahn and Hevesy (6), the inhibitory effect of cyanide on the entry of P^{32} into the cell can be explained in terms of decreased esterification of inorganic phosphate. The same explanation probably applies to other inhibitors (4, 9).

In interpreting the experiments in which Krebs–Ringer was substituted for plasma as the suspending medium, it should be remembered that the P^{31} concentration in the Krebs–Ringer – bicarbonate approximates that of plasma, whereas that of the Krebs–Ringer – phosphate solution is much greater (by a factor of more than 9). It might be reasonable to assume, that in the presence of a higher concentration of phosphate in the suspending medium, the P^{32} would be incorporated into the cells at a slower rate. However, for rabbit cells suspended in plasma, Hahn and Hevesy (6) found little change in the rate of penetration of P^{32} when the phosphate concentration was increased by a factor of eight, and Eisenman, Ott, Smith, and Winkler (3) reported a similar result for human cells.

The percentage of the added P^{32} that was incorporated into the red cells of normal rabbits in a given time was greater than that reported by Hahn and Hevesy (6) for rabbit blood, and was also greater than the figures of Eisenman *et al.* (3), Taylor, Levenson, and Adams (10), and Gemmill and co-workers (5, 9) for human blood. All these workers used whole blood. The experiments reported here were done on red cell suspensions with a haematocrit in the vicinity of 50%, a condition that would favor the incorporation of a greater percentage of the added P^{32}.

Acknowledgments

Mr. C. S. Sanborn and Mr. S. M. J. Richardson determined the concentration of lipids in the plasma.

References

1. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H. Federation Proc. 10: 18. 1951.
2. Burt, N. S., Murray, R. G. E., and Rossiter, R. J. Blood, 6: 906. 1951.
3. Eisenman, A. J., Ott, L., Smith, P. K., and Winkler, A. W. J. Biol. Chem. 135: 165. 1940.
4. Gourley, D. R. H. Am. J. Physiol. 164: 213. 1951.
5. Gourley, D. R. H. and Gemmill, C. L. J. Cellular Comp. Physiol. 35: 341. 1950.
6. Hahn, L. and Hevesy, G. Acta Physiol. Scand. 3: 193. 1942.
7. Hevesy, G. *In* Radioactive indicators. Interscience Publishers Inc., New York. 1948.
8. London, I. M., Shemin, D., and Rittenberg, D. J. Biol. Chem. 183: 749. 1950.
9. Pertzoff, V. and Gemmill, C. L. J. Pharmacol. Exptl. Therap. 95: 106. 1949.
10. Taylor, F. H. L., Levenson, S. M., and Adams, M. A. Blood, 3: 1472. 1948.
11. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. *In* Manometric techniques and related methods for the study of tissue metabolism. Burgess Publishing Co., Minneapolis. 1945.

SYNERGISTIC ACTION OF ANTABUSE[R] AND INTRAVENOUS CYANAMIDE ON BLOOD ACETALDEHYDE FOLLOWING ALCOHOL ADMINISTRATION[1]

By Floyd R. Skelton, Hilda M. McConkey, and Gordon A. Grant

Abstract

The effect of pretreatment with Antabuse[R] and intravenous cyanamide on the blood concentration of acetaldehyde following alcohol administration has been studied in rabbits. Both Antabuse[R] and intravenous cyanamide when administered separately produced an abnormal accumulation of acetaldehyde in the blood under these conditions. Combined pretreatment with Antabuse[R] and intravenous cyanamide resulted in a blood level of acetaldehyde greater than either substance produced alone and greater than could be accounted for on an additive basis. It is concluded that some synergistic action seems to exist between Antabuse[R] and intravenous cyanamide as measured by the blood acetaldehyde concentrations obtained after alcohol injection.

Introduction

Acetaldehyde is well known to be a normal metabolite of alcohol in both animals and man (2, 12, 13). The concentration of acetaldehyde in the blood has been shown to increase greatly after alcohol administration when the organism was pretreated with Antabuse[R]* (5, 10). It is the abnormal accumulation of acetaldehyde which apparently is responsible for the characteristic signs and symptoms of the so-called "Antabuse[R] alcohol reaction"(1).

It has been noted by numerous investigators that similar effects follow alcohol ingestion by people working in contact with cyanamide (3, 4, 7, 9, 11, 14). Hald, Jacobsen, and Larsen (6) have recently shown that intravenous cyanamide, like Antabuse[R], sensitized the rabbit to alcohol and resulted in a greatly increased blood acetaldehyde concentration. The experimental results contained in the present publication further suggest that a synergistic action on alcohol metabolism exists between Antabuse and intravenous cyanamide.

Materials and Methods

Rabbits weighing 3-4 kgm. were used throughout as the experimental animal. Antabuse[R] was administered per gavage as a 20% suspension in corn oil daily for three days, the last dose being given four hours before the beginning of the experiment. Cyanamide was injected intravenously 30 min. prior to the beginning of the experiment. A cannula was inserted into one carotid artery under sodium pentobarbital anesthesia to allow withdrawal of 5-ml.

[1] *Manuscript received December 14, 1951.*
Contribution from the Research and Biological Laboratories, Ayerst, McKenna, and Harrison, Ltd., Montreal, Can.

** Antabuse [R] is the registered trade-mark of Ayerst, McKenna, and Harrison Limited and denotes an especially prepared and highly purified form of tetraethylthiuram disulphide:*

$$(C_2H_5)_2\ N-\overset{\overset{S}{\|}}{C}-S-S-\overset{\overset{S}{\|}}{C}-N\ (C_2H_5)_2.$$

blood samples at frequent intervals. Blood clotting was prevented by heparinization. Five minutes after the first blood collection, 30 ml. of 10% ethanol were given intravenously over a three-four minute interval. Further samples were withdrawn at 10 min. intervals for 50 min. after the first sample and a final collection was made at 80 min. The acetaldehyde content of the blood samples was determined by the method of Stotz (12).

Two rabbits without pretreatment served as controls. Groups of two animals received the following pretreatment in the manner outlined above: Antabuse^R alone in doses of 0.75 gm. and 0.2 gm. daily for three days; cyanamide alone in doses of 0.2 gm. per kgm. and 0.05 gm. per kgm; Antabuse^R in doses of 0.2 gm. daily for three days plus cyanamide, 0.05 gm. per kgm; Antabuse^R in doses of 0.4 gm. daily for three days plus cyanamide, 0.1 gm. per kgm.

Results

The acetaldehyde concentration in the blood samples taken prior to ethanol administration showed that Antabuse^R or cyanamide alone, or in combination, did not alter the normal level of this metabolite. The control animals receiving only ethanol showed a slight rise in blood acetaldehyde, the highest value being 0.27 mgm. per 100 ml. of blood (Figs. 1 and 2). Pretreatment with

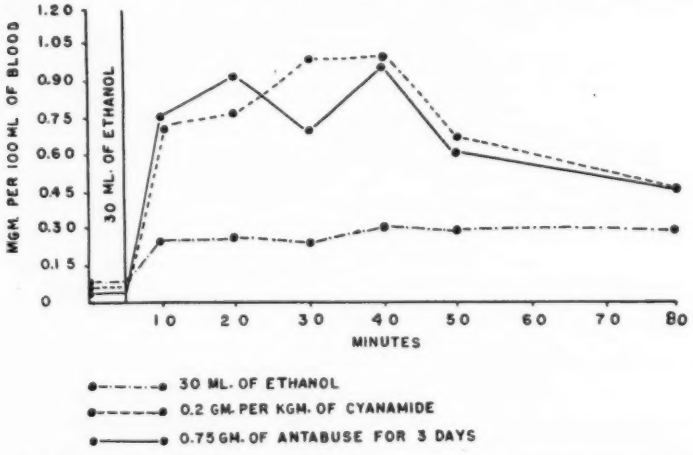


FIG. 1. The effect of Antabuse^R and intravenous cyanamide pretreatment on the blood levels of acetaldehyde after ethanol administration in the rabbit.

0.75 gm. of Antabuse^R daily for three days, or a single intravenous injection of 0.2 gm. per kgm. of cyanamide, produced a marked acetaldehyde accumulation in the blood following ethanol administration. As seen in Fig. 1 the highest values obtained were 91 and 95 mgm. per 100 ml. of blood respectively.

When the dose of Antabuse^R was reduced to 0.2 gm. daily and cyanamide to 0.05 gm. per kgm., the blood acetaldehyde level obtained after ethanol

administration was only slightly greater than that found in the nonpretreated control animals (Fig. 2). However, when combined pretreatment with these substances was given at the same dosage, the highest blood acetaldehyde

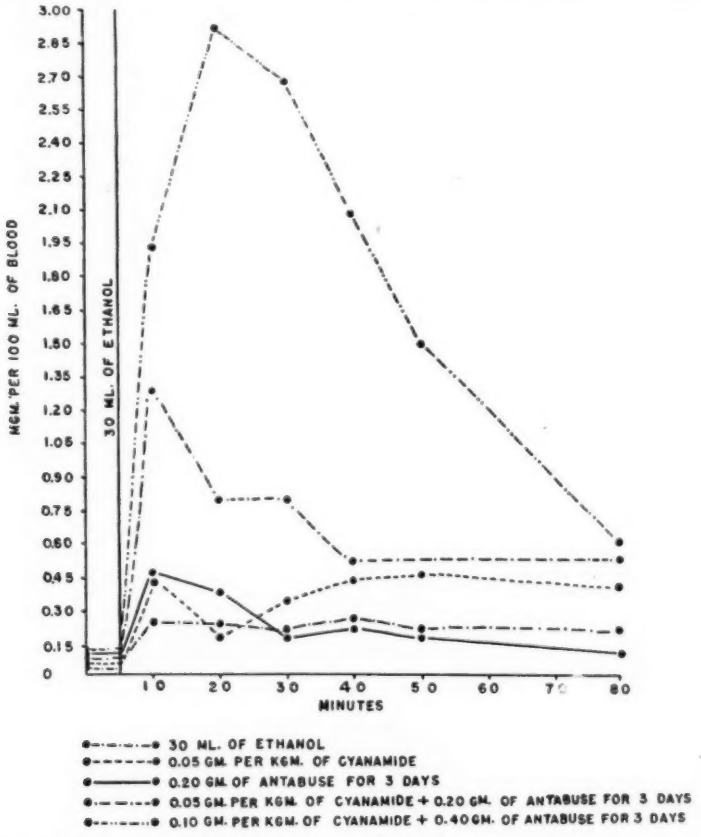


FIG. 2. The comparative effects of pretreatment with Antabuse[R] and intravenous cyanamide, alone and in combination, on the blood levels of acetaldehyde after ethanol administration in the rabbit.

levels developed after ethanol injection averaged 1.28 mgm. per 100 ml. of blood (Fig. 2). This concentration of acetaldehyde was greater than that observed when Antabuse[R] or cyanamide was given alone in doses four times larger (Fig. 1) and appears to be greater than can be explained by a simple additive effect. Doubling of the dose of Antabuse[R] and cyanamide to 0.4 gm. and 0.1 gm. per kgm. respectively, and given as combined pretreatment, resulted in blood acetaldehyde levels which were four times (Fig. 2) those obtained with twice the dose of these substances when each was given alone (Fig. 1).

Discussion

These studies confirm the sensitizing effect of Antabuse[R] and intravenous cyanamide to alcohol as shown by the abnormal accumulation of acetaldehyde in the blood. While the increased blood concentration of this metabolite is common, the mechanism by which it is produced appears to be different, since Kjeldgaard (8) has shown that Antabuse[R] inhibited the oxidation of aldehydes to acids by liver aldehyde oxidase while cyanamide even in high concentration was without effect. Regardless of the apparent differences in method of action on alcohol metabolism a synergistic effect seems to exist between Antabuse[R] and intravenous cyanamide in causing an elevation in the blood acetaldehyde concentration.

References

1. Asmussen, E., Hald, J., and Larsen, V. Acta Pharmacol. Toxicol. 4: 311. 1948.
2. Briggs, A. P. J. Biol. Chem. 71: 67. 1926.
3. De Gaulejac, R. and Dervillee, P. Arch. maladies profess. hyg. et toxicol. ind. 6: 323. 1945.
4. Gartner, N. Münch. med. Wochschr. 90: 1745. 1939.
5. Hald, J. and Jacobsen, E. Acta Pharmacol. Toxicol. 4: 305. 1948.
6. Hald, J., Jacobsen, E., and Larsen, V. Arch. maladies profess. méd. travail et sécurité sociale, 10: 232. 1949.
7. Hesse, E. Z. ges. exptl. Med. 25: 321. 1921.
8. Kjeldgaard, N. O. Acta Pharmacol. Toxicol. 5: 397. 1949.
9. Koelsch, F. Münch. med. Wochschr. 61: 1869. 1914.
10. Larsen, V. Acta Pharmacol. Toxicol. 4: 321. 1948.
11. Mellinghoff, K. and Thomas, O. Deut. med. Wochschr. 65: 1636. 1939.
12. Stotz, E. J. Biol. Chem. 148: 585. 1943.
13. Supnievski, J. V. J. Biol. Chem. 70: 13. 1926.
14. Thiry, U. Arch. maladies profess. hyg. et toxicol. ind. 4: 132. 1942.

PRODUCTION EN SAC DE CELLOPHANE DE TOXINES TÉTANIQUES RENFERMANT AU MOINS 600,000 DOSES MORTELLES (COBAYE) PAR MILLILITRE[1]

PAR V. FREDETTE ET G. VINET

Abstract

By cultivating the Pasteur Institute strain of *Clostridium tetani* in cellophane bags immersed in Weinberg's *Vf* broth, toxins containing from 600,000 to 1,200,000 guinea pig minimum lethal doses per milliliter have been obtained. Without the cellophane, the same strain and the same medium gave not more than 100,000 guinea pig M.L.D. per milliliter.

Au cours des dix dernières années, la technique de Prévot (4) de l'Institut Pasteur a permis d'obtenir dans nos laboratoires des toxines tétaniques renfermant en général de 100,000 à 200,000 doses mortelles (cobaye) par millilitre en lots de 150-200 litres par semaine (1).

Dans le but d'améliorer cette production, nous avons tenté d'adapter à la toxine tétanique la technique utilisée par Sterne et Wentzel (6) pour la toxine botulinique.

Technique et Résultats

Un tube de cellophane Visking de 2 pouces de diamètre par 9 pouces de longueur est suspendu dans 12 litres de bouillon *Vf* (5); on remplit la cellophane avec de l'eau salée à 8.5 p. 1000 et on stérilise le tout comme à l'ordinaire. Après refroidissement, on ensemence en portant la culture de *Clostridium tetani* (souche Institut Pasteur) non pas dans le bouillon mais dans l'eau salée où ont déjà diffusé des substances nutritives. Après 11-14 jours à l'étuve réglée à 33°C., on obtient des taux de croissances inaccoutumés et des toxines dont voici les titres:

Lot No	Sans cellophane	Avec cellophane
447	<100,000 dmm./ml.	600,000 dmm./ml.
450	100,000 dmm./ml.	1,200,000 dmm./ml.
455	<100,000 dmm./ml.	1,000,000 dmm./ml.
462	<100,000 dmm./ml.	800,000 dmm./ml.
462*a*	<100,000 dmm./ml.	800,000 dmm./ml.

Ces résultats très encourageants ont conduit à la réalisation d'un appareil (7) qui a permis d'obtenir des toxines tétaniques naturelles d'activité jusqu'ici inconnue: 2,000,000 dmm. (cobaye) par ml. et 350 unités floculantes. Sans tube de cellophane, le même milieu et la même souche n'ont fourni que 100,000 dmm. par ml.

[1] *Manuscrit reçu le 21 septembre 1951.*

Contribution de l'Institut de Microbiologie et d'Hygiène de l'Université de Montréal, Montréal, Qué.

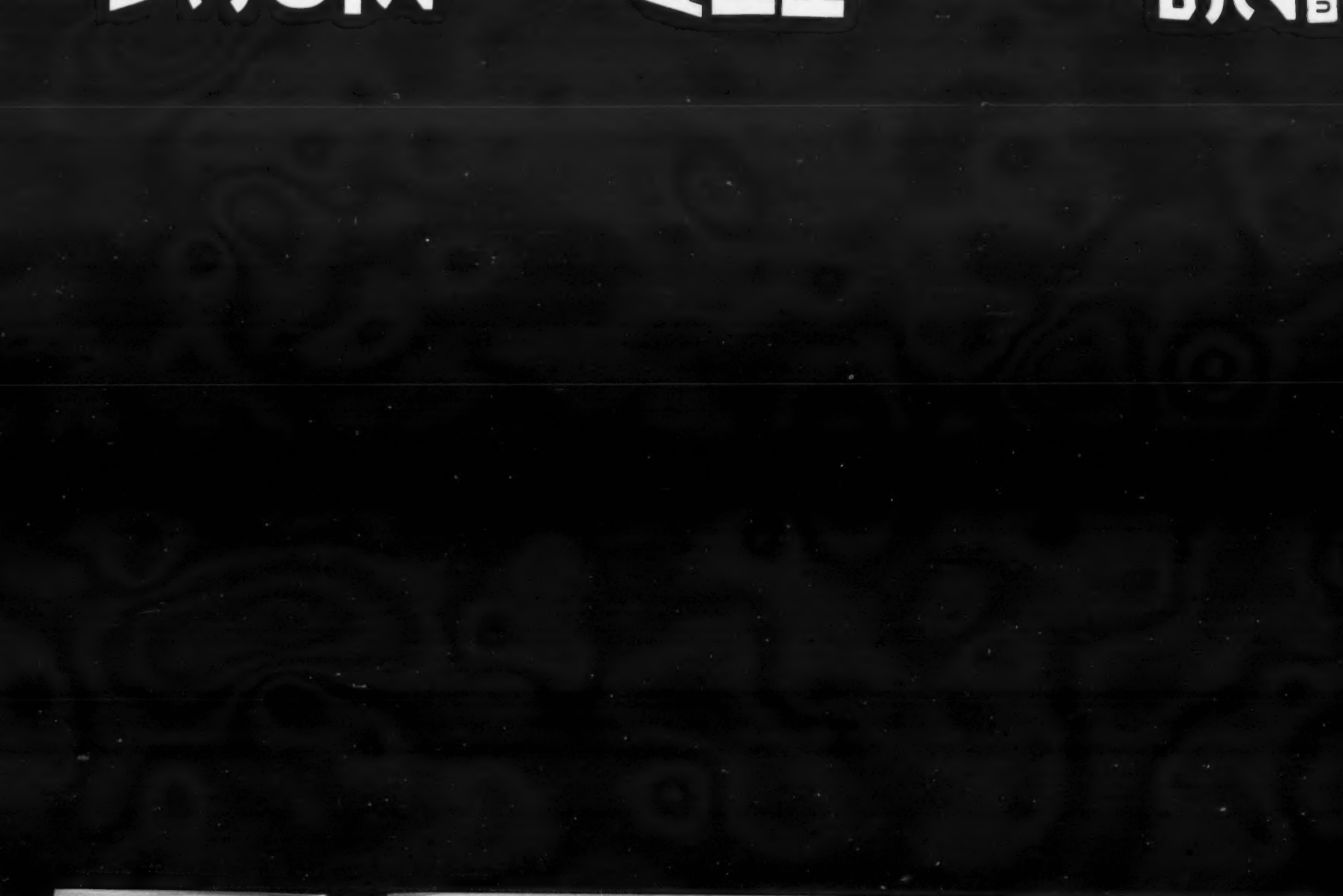
Commentaires

La toxicité de ces filtrats dépasse d'emblée celle des meilleures toxines fabriquées avec des milieux de culture synthétiques (3). C'est dire que les milieux empiriques restent encore les plus propres à la production de toxines naturelles hautement actives puisqu'ils renferment des facteurs stimulants qui échappent à l'analyse.

Ces premières indications laissent évidemment espérer des résultats pratiques intéressants mais ils comportent aussi un intérêt théorique considérable. En effet, la culture en tube de cellophane semble allonger considérablement la phase de multiplication bactérienne; il est permis de supposer alors que cette méthode réalisera la culture continue à taux limité avec une installation beaucoup plus simple que celle proposée récemment par Monod (2).

Bibliographie

1. FREDETTE, V. Douze ans de progrès. Institut de Microbiologie et d'Hygiène de l'Université de Montréal. 1950.
2. MONOD, J. Ann. inst. Pasteur, 79: 390. 1950.
3. MUELLER, H. J. and MILLER, P. A. J. Immunol. 50: 377. 1945.
4. PRÉVOT, A.-R. Compt. rend. 208: 394. 1939.
5. PRÉVOT, A.-R. Communication personnelle.
6. STERNE, M. and WENTZEL, L. M. J. Immunol. 65: 175. 1950.
7. VINET, G. et FREDETTE, V. Science, 114: 549. 1951.



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